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Loss of CD20 Expression after Rituximab Therapy for B-Cell Lymphomas: A Review of the Literature

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Abstract

Rituximab (Rx), a chimeric anti-human CD20 antibody, is used widely for the treatment of B-cell non-Hodgkin's lymphomas (NHL) worldwide. Loss of CD20 expression in relapsed B-cell lymphomas after Rx treatment, however, is observed in some cases, which might be a cause of B-cell NHL unresponsiveness to Rx retreatment. The frequency of loss of CD20 expression after Rx treatment and radiotherapy, its correlation with histological changes, and its clinical implication together with possible molecular mechanisms are discussed in this review of pertinent literature. In high-grade B-cell NHL, loss of CD20 expression after Rx treatment was observed less frequently in Japan than in Australia. Evaluation of CD20 expression by immunohistochemical and flow cytometric methods is a reliable guide for employment of Rx treatment for B-cell lymphomas.

Keywords: B-cell lymphomas, high-grade, low-grade, rituximab

1. Introduction

CD20, a hydrophobic transmembrane protein with a molecular weight of approximately 35 kD, is expressed in pre B and mature B lymphocytes (Nadler et al., 1981). Rituximab (Rx) is a chimeric anti-human CD20 antibody that is used widely for the treatment of B-cell lymphomas (Reff et al., 1994) and immune-related diseases, such as rheumatoid arthritis (Edwards et al., 2006). The mechanisms of action of Rx for elimination of non-neoplastic and neoplastic B-cells include antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity, and stimulation of the apoptotic pathway (Reff et al., 1994). Rx was employed originally for the treatment of low-grade B-cell lymphomas or follicular lymphoma (FL). Later, the combined use of Rx with conventional chemotherapy (cyclophosphamide, doxorubicin, vincristine, and prednisone) (Rx-CHOP) was found to be effective for more aggressive diffuse large B-cell lymphoma (DLBCL) (Feugier et al., 2005).

DLBCL, the most common type of malignant lymphoma worldwide, is a diffuse proliferation of large neoplastic B-lymphoid cells. On the basis of gene expression profiles, DLBCL can be categorized into two distinct subtypes: germinal center B-cell (GCB) and activated B-cell (ABC), or non-GCB (Alizadeh et al., 2000). Generally, the non-GCB type is associated with an unfavorable prognosis compared to the GCB type before the employment of Rx therapy.

Positive regulatory domain 1 (PRDM1), a master regulator of the differentiation of mature B lymphocytes into plasma cells, has two isoforms, PRDM1-alpha and -beta, the expressions of which are regulated by the transcriptional regulator NF-kappaB. Using microdissected DLBCL cells, Liu et al. reported that both PRDM1-alpha and -beta were expressed in the non-GCB, but not the GCB, type of DLBCL (Liu et al., 2007). Expression of the PRDM1-beta gene was shown to be correlated with an unfavorable prognosis of non-GCB patients when treated with CHOP, but this was not observed in those undergoing Rx-CHOP treatment (Liu et al., 2007), suggesting a favorable effect of Rx. This same study also reported that B-lymphoma cells resistant to chemotherapy expressed PRDM1-beta, and this expression was suppressed by Rx, possibly through NF-kappaB inactivation. Thus, expression of PRDM1-beta could be a prognostic marker for the non-GCB type of DLBCL.

Rx-CHOP is now employed as a standard therapy for DLBCL, but recurrence of disease is encountered not infrequently. In such cases, histologic examination of relapsed tumors is not usually performed because DLBCL does not transform to more become more, or less, aggressive. Therefore, reports on the loss of CD20 expression after Rx therapy for B-cell non-Hodgkin's lymphomas (NHL) have been relatively limited.

Tumors occasionally become resistant to therapies that are initially effective. The same phenomenon is observed in B-cell NHL treated with Rx-containing chemotherapy, followed by reduced responsiveness of lymphoma cells to the therapy; loss or reduction of CD20 in lymphoma cells is one cause. In this paper, changes in CD20 expression in B-cell NHL after Rx therapy are discussed with respect to clinical behavior and molecular mechanisms. Davis et al. reported that the response rate of relapsed B-cell NHL to Rx after Rx therapy was less than 50% (Davis et al., 2000). In such cases, loss of CD20 expression in relapsed B-cell lymphomas could be a robust indicator of resistance to Rx therapy.

2. Resistance to Rx Therapy in B-cell Lymphomas

Possible mechanisms of the resistance of B-cell NHL to Rx therapy include three patterns: protection of the tumor cells from Rx-triggered elimination by ADCC / complement-dependent cytotoxicity and apoptotic stimulation, inadequate binding of Rx to the CD20 molecule, and loss of CD20 expression.

2.1 Antibody-Dependent Cellular Cytotoxicity (ADCC)

ADCC by natural killer (NK)-cells plays a major role in elimination of B-lymphoma cells during Rx therapy. Rx-induced ADCC was attenuated upon ligation of killer immunoglobulin-like receptors, inhibitory receptors expressed on NK-cells, by human leukocyte antigen (HLA) molecules expressed on human B-lymphoma target cells (Borgerding et al., 2010). Therefore, protection of tumor cells from ADCC by inhibition of NK-cell function through increased HLA expression on tumor cells might explain the failure of Rx treatment for CD20 positive B-cell NHL.

2.2 Binding of Rx to CD20

Inadequate binding of Rx to the CD20 molecule might be caused by mutations or polymorphisms of the CD20 gene that affect its structure. However, Sar et al. reported that no mutations were detected in the coding region of the CD20 gene in any of 11 patients with DLBCL who showed a poor prognosis with Rx-CHOP therapy (Sar et al., 2009); one case showed a synonymous single nucleotide polymorphism in exon 2. Johnson et al. reported similar results, demonstrating that mutations of the Rx epitope in the CD20 gene, encompassing exon 5 of the *MS4A1* gene, were detected in only one of 264 (0.4%) or one of 15 (6%) biopsies taken at diagnosis or relapse, respectively (Johnson et al., 2009a). No polymorphic sequence variants were detected in this region. Taken together, CD20 mutations involving the Rx epitope are rare in both *de novo* and relapsed DLBCL.

2.3 Loss of CD20 Expression after Rx Therapy for B-cell NHL

Loss of CD20 expression after Rx therapy for B-cell lymphomas is observed as a consequence of purging CD20-expressing B-lymphoma cells. Putative mechanisms are described below.

2.3.1 Mutations of the CD20 Gene

Terui et al. reported that deletion mutations in the C-terminus of CD20 were found in 4/50 (8.0%) cases of B-cell lymphomas; 2/22 cases of DLBCL, 1/7 of FL, 1/1 of mantle cell lymphoma, and 0/20 of other B-cell lymphomas (Terui et al., 2009). This resulted in decreased mean fluorescence intensity of CD20 expression on fresh lymphoma cells compared to cells with non-mutated genes. Three of their 44 (6.8%) patients who had received Rx-CHOP therapy had C-terminal deletion mutations, and they showed progressive disease after Rx-CHOP therapy, suggesting a role for mutation in disease progression. Radiotherapy was employed in two of Terui et al.'s cases before administration of Rx, and deletion of the C-terminus of the CD20 gene and disease progression were found in these cases. Radiation before Rx administration might also cause mutation of the CD20 gene. On the contrary, effects of the CD20 gene sequence on the level of CD20 protein expression were not found in other studies (Tomita et al., 2007; Czuczman et al., 2008). Tomita and colleagues (Tomita et al., 2007) reported epigenetic regulation of CD20 expression in a CD20-negative mature B-cell line, RRBL1, established from a patient treated repeatedly with Rx-containing chemotherapy.

The Gene Scan analysis in our study revealed partial or complete persistence of the same-sized peaks in 11 DLBCL cases, indicating the same origin of tumor cells before and after Rx-containing therapy; however, changes in the peak pattern were also found in many cases, suggesting the presence of genetic instability (Figure 1) (Wada et al., 2009). These findings may explain partly the occurrence of CD20-negative DLBCL after Rx-containing therapy.

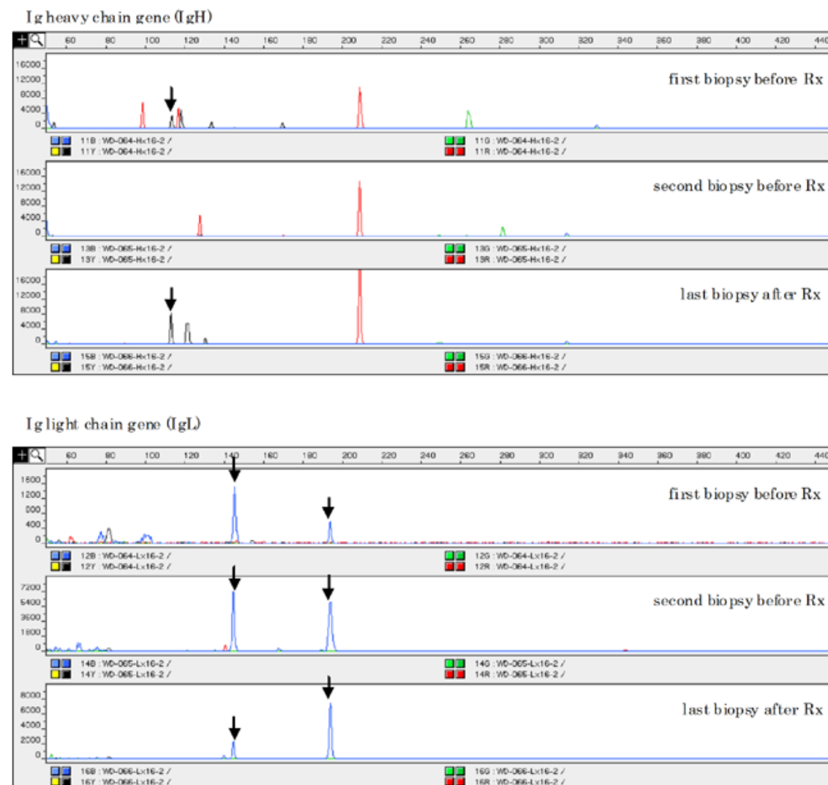

Figure 1. Polymerase chain reaction-based clonality analysis of immunoglobulin (Ig) gene rearrangement (Gene Scan analysis) in a case of diffuse large B-cell lymphoma revealed different peak patterns before and after rituximab (Rx) treatment, with partial persistence of the same-sized peaks (↓)

2.3.2 Immunohistochemical (IHC) and Flow Cytometric (FCM) Analysis

Johnson et al. reported that tumor cells in 43 of 272 (16%) DLBCL cases showed reduced CD20 expression, 35 of whom also exhibited bright CD19 expression (Johnson et al. 2009b). These 35 cases had a worse prognosis than the other cases with bright CD20 expression when treated with CHOP or Rx-CHOP, irrespective of the international prognostic index. Forty-one of the 43 cases with reduced CD20 expression by FCM showed strong staining for CD20 by IHC. Sequencing of exon 5 of the *MS4A1* gene, encoding the extracellular component of the CD20 antigen, did not reveal mutations that could explain the discrepant results between FCM and IHC. Cases showing loss of CD20 expression by FCM, but which were positive for CD20 by IHC, in recurrent tumors of DLBCL after Rx treatment were also reported (Wada et al., 2009; Kennedy et al., 2002); these cases showed progressive disease during Rx-containing therapy. These findings suggest that lack of detection of CD20 by FCM is a sign of resistance to Rx-containing therapy. FCM and IHC analyses detect different epitopes of CD20; extracellular surface epitopes are detected by FCM whereas intracellular ones are detected by IHC. Masking of the surface epitopes through binding of CD20 molecules, instead of gene mutations that affect surface epitopes but preserve intracellular ones, might occur.

2.3.3 Rx-resistant Cell Lines (RRCL) as a Model for Loss of CD20 Expression

Tsai et al. reported that rituximab-resistant cell lines (RRCL) exhibited a gradual loss of CD20 surface expression through repeated exposure to Rx (Tsai et al., 2012). They found that the promoter activity of the CD20 gene was decreased, due to reduced binding of several key positive regulatory proteins on the CD20 promoter. Forced CD20 expression restored cytoplasmic, but not surface, CD20, suggesting a defect in CD20 protein transport. Thus, addition of interleukin-4 (IL-4) might induce higher CD20 promoter activity and CD20 expression, improving the responsiveness of RRCL to Rx.

3. Clinical Implications of Loss of CD20 Expression in B-Cell Lymphomas after Rx Therapy

3.1 Histological Changes and CD20 Expression (Table 1)

Low-grade B-cell NHL-expressing CD20 might recur as high-grade CD20-negative NHL, which could explain, at least in part, the resistance of recurrent tumors to Rx retreatment (Davis et al., 1999; Schmitz et al., 1999; Alvaro-Naranjo et al., 2003; Maeshima et al., 2009; Hiraga et al., 2009). However, Foran et al. reported that loss of CD20 expression after Rx treatment was not correlated with histological transformation from low-grade to high-grade NHL (Foran et al., 2001).

Table 1. Frequency of loss or significant decrease of CD20 expression after rituximab treatment for CD20-positive B-cell lymphomas

	Frequency of loss or significant decrease of CD20 expression (%)	Histological change (case no.)	Authors/Year
DLBCL	0/1 (0)	NA	Seliem et al/2006
	4/11 (36.4)	no remarkable change (3) proliferation of plasmacytoid cells (1)	Maeshima et al/2009
	3/7 (42.9)	NA	Hiraga et al/2009
	4/21 (19.0)	no remarkable change (4)	Wada et al 2009
MZBCL	0/2 (0)	NA	Seliem et al/2006
	1/2 (50)	proliferation of plasmacytoid cells (1)	Maeshima et al/2009
	0/2 (0)	NA	Hiraga et al/2009
CLL/SLL	3/4 (75)	NA	Seliem et al/2006
	2/2 (100)	no remarkable change (2)	Maeshima et al/2009
FL	9/34 (26.5)	no remarkable change (5) proliferation of plasmacytoid cells (2) transformation to Hodgkin's lymphoma (1) transformation to anaplastic large cell lymphoma-like undifferentiated lymphoma (1)	Maeshima et al/2009
	2/7 (28.6)	transformation to DLBCL (2)	Hiraga et al/2009
MCL	0/10 (0)	NA	Maeshima et al/2009
	0/1 (0)	NA	Hiraga et al/2009
LPL	1/1 (100)	NA	Seliem et al/2006
Burkitt or Burkitt-like	0/2 (0)	NA	Hiraga et al/2009
aggressive B-NHL*	6/10 (60)	NA	Kennedy et al/2002
B-NHL, NOS	1/1 (100)	NA	Seliem et al/2006

*DLBCL and MCL (mainly DLBCL)

DLBCL indicates diffuse large B-cell lymphoma; MZBCL, marginal zone B-cell lymphoma; CLL/SLL, chronic lymphocytic leukemia/small lymphocytic lymphoma; FL, follicular lymphoma; MCL, mantle cell lymphoma; LPL, lymphoplasmacytic lymphoma; B-NHL, B-cell non Hodgkin's lymphoma; NOS, not otherwise specified; NA, data not available

3.2 Transient Loss of CD20 Expression

Ferreri et al. reported transient loss of CD20 expression in a case of gastric DLBCL after Rx-containing treatment; the tumor cells were initially CD20-positive, turned negative at the first relapse, and restored CD20 expression at the second relapse (Ferreri et al., 2007). Because loss of CD20 expression could be a transient phenomenon, it is meaningful to evaluate CD20 expression at every relapse of tumors to inform decisions regarding Rx-containing regimens.

3.3 Frequency of Loss of CD20 Expression after Rx Therapy for Low- and High-Grade B-Cell NHL (Table 1)

Loss or a significant decrease in CD20 expression was found in various kinds of B-cell NHL and was relatively common in cases with CLL/SLL in previous reports (Seliem et al., 2006; Maeshima et al., 2009). There was no correlation between loss of CD20 expression and interval of biopsies, treatment modalities, clinical response, or frequency and dose of Rx therapy (Maeshima et al., 2009). Jilani et al. reported down-regulation of CD20 expression at the RNA level after exposure of CLL cells to Rx (Jilani et al., 2003). Thus, evaluation of CD20 expression might identify a subset of CLL/SLL patients who will not benefit from repeated therapy with Rx.

Kennedy et al. reported that loss of CD20 expression after Rx treatment was frequently observed in cases of DLBCL, which resulted in progressive disease (Kennedy et al., 2002). A lower frequency, 4 of 21 cases (19%), of loss of CD20 expression after Rx treatment for DLBCL was reported from Japan (Figure 2) (Wada et al. 2009). The mean Rx dose administered until CD20-negative relapse in the report of Kennedy et al. (5 doses) was lower than that in our cases (9 doses), but the difference was not significant (Wada et al., 2009). The mean time between the last administration of Rx and CD20-negative relapse in the Kennedy et al. cases (5.3 months) was similar to that of our cases (4.5 months).

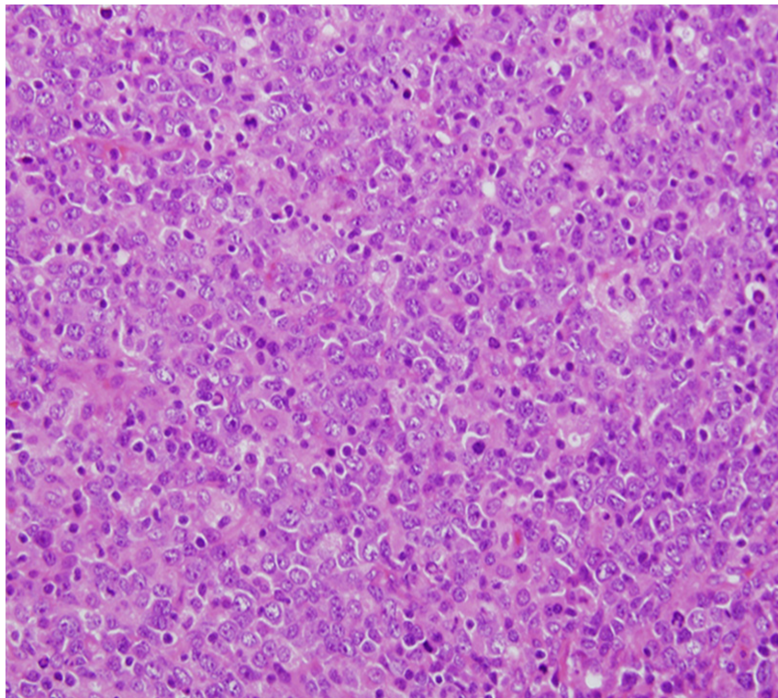

Figure 2. A. Initial diffuse large B-cell lymphoma (DLBCL) (one of our cases). H&E

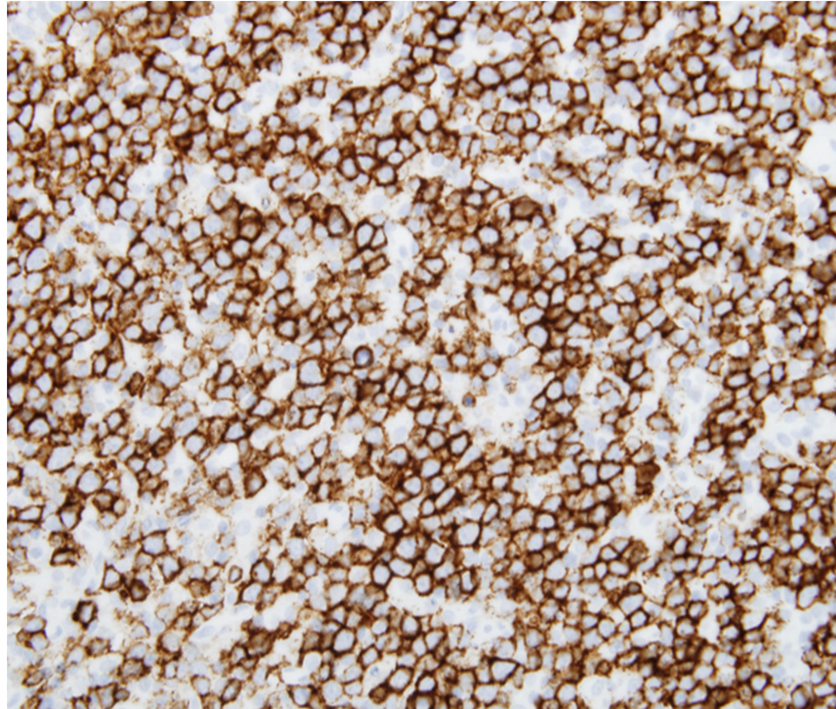

Figure 2. B. Tumor cells were $CD20^{+}$ by immunohistochemistry

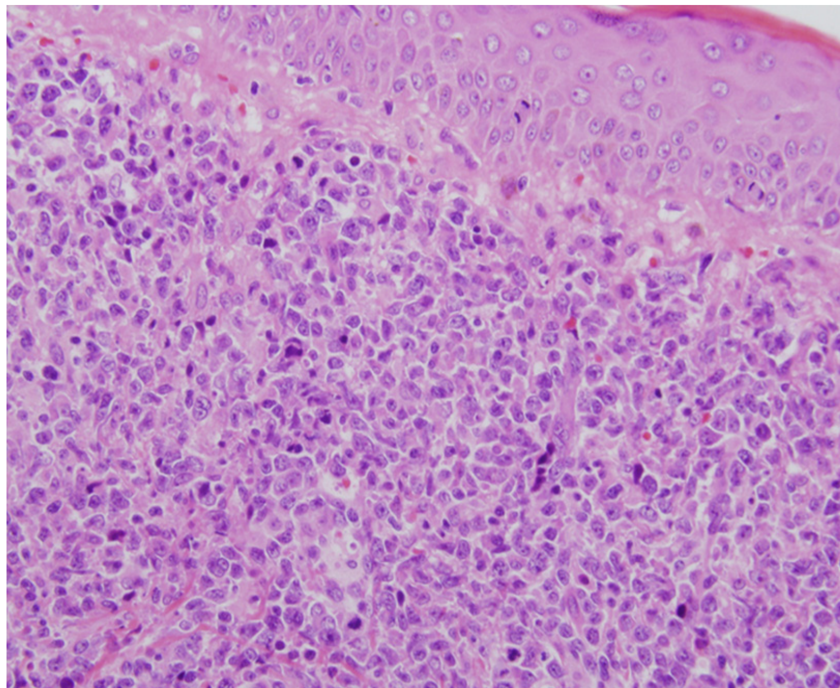

Figure 2. C. Recurrent DLBCL after rituximab treatment showed similar features to the initial DLBCL. H&E

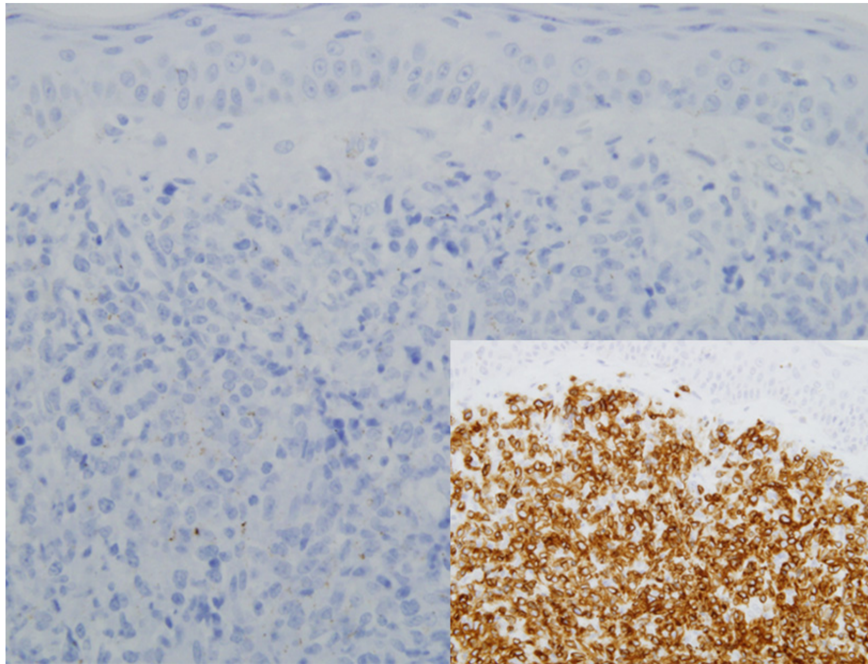

Figure 2. D. Recurrent tumor cells were CD20^{-}. Inset: tumor cells were CD79a^{+}. Magnification, x400

4. Conclusion

Loss of CD20 expression in neoplastic B-cells could be a cause of B-cell NHL unresponsiveness to Rx-containing chemotherapy, which might result in an unfavorable prognosis. Therefore, estimation of CD20 expression is a prerequisite for employment of Rx therapy for B-cell NHL.

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Cytokine and Chemokine Expression Profiles in HIV-1 Infected Patients with Ocular Surface Squamous Neoplasia from Botswana

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Abstract

Purpose: Ocular surface squamous neoplasia (OSSN) rate has increased in incidence with the HIV pandemic in Africa. Multiple factors including cellular and environmental can affect the pathogenesis of OSSN in HIV-infected patients. We will investigate anti-inflammatory cytokines, proinflammatory cytokines, and growth factor expression in sera and tissue samples of OSSN and pterygia for the potential link to the development of OSSN. **Results:** Antibody analysis showed significant changes in levels of pro-inflammatory cytokines, anti-inflammatory cytokines and growth factors in sera. Quantitative RT-PCR of tissues showed expression of inflammatory cytokines and chemokines associated with HIV infection and carcinogenesis. **Conclusion:** Our findings showed that dysregulation in expression of cytokines and growth factors in patients with multiple infections may contribute to pathogenesis of OSSN and pterygia. The data reinforces the significance of in depth analysis of immune function in HIV-1 OSSN patients with multiple viral infections that has potential for therapy and vaccine development.

Keywords: cytokine, OSSN, pterygium, HIV-1, immunosupression

1. Introduction

Ocular surface squamous neoplasia (OSSN) is a conjunctival or corneal neoplastic growth that covers dysplasia, to conjunctival intraepithelial neoplasia (CIN) and invasive squamous cell carcinoma (Kiire & Dhillon, 2006). Similar to cancer of the cervix, its rate of recurrence after treatment is increasing it and may metastasize in some patients, but in poor countries recorded recurrence is still low (Waddell, Downing, Lucas, & Newton, 2006). Recently there has been a strong association of OSSN with the HIV pandemic, and colinearity in incidence with HIV-1infection has been observed except in Uganda where a decrease has been noted with the decrease in HIV-1 incidence (Wabinga, Parkin, Wabwire-Mangen, & Nambooze, 2000; Maxwell, Parkin, Nambooze, Wabwire-Mangen, & Wabwinga, 2010). Literature indicates that preceding the HIV-1 pandemic, OSSN predominantly occurred in the elderly for whom it is the third most common oculoorbital tumor after melanoma and lymphoma (Maxwell et al., 2010; de Koning et al., 2008). Other risk factors linked to its pathogenesis have included ultraviolet light B rays (de Koning et al., 2008), mutation of the p53 tumor suppressor gene (Tornesello, Waddell, Duraturo, Biryahwaho, Downing, & Lucas, 2005), immunosuppression in organ transplant recipients (Vajdic et al., 2007), cigarette smoking, and in some settings, human papillomavirus (HPV) infection (de Koning

et al., 2008; McDonnell, Mounts, Wu, & Green, 1986). In sub-Saharan Africa, OSSN is increasing in prevalence, aggressiveness, and affects predominantly young people who are HIV-1 positive with a greater percentage of them being women (Waddell et al., 2006; Tornesello et al., 2005).

Pterygium has been described as a benign growth on the conjunctiva often associated with over-exposure to the sun (Panchapakesan, Hourihan, & Mitchell, 1998). Dry dusty conditions may also be a contributing factor (Panchapakesan et al., 1998). Some studies have investigated HPV, HSV, and EBV as possible risk factors in development of pterygia (Tsai et al. 2009; Hirst, Axelsen, & Schwab, 2009; Piecyc-Sidor, Polz-Dacewicz, Zagorski, & Zarnowski, 2009; Piras et al., 2003; Gallagher, Giannoudis, Herrington, & Hiscott, 2001; Detorakis, Sourvinos, & Spandidos, 2001; Varinli, Koksal, & Doran, 1994). Additionally, in our recent study we identified oncogenic herpes viruses associated with both OSSN and pterygia suggesting additional biological agents as cofactors which may contribute to the disease (Simbiri et al., 2010).

Cytokines and chemokines are significant factors in innate immunity, apoptosis, angiogenesis, cell growth, and differentiation (Chopra, Dinh, & Hannigan, 1998). They are involved in a vast array of diseases including cancer, and can enhance or modulate these processes by their inflammatory activities (Chopra et al., 1998). Inflammatory cytokines and chemokines such as TNF-α, TGF-β, IFN- , IL-1β, IL-6, and IL-8 were shown to be involved in inflammatory activities associated with development of HPV linked cancers (Chopra et al., 1998). Table 1 shows some of the cytokines, chemokines, and growth factors in sera commonly reported to be dysregulated in HIV patients.

Table 1. Cytokines, chemokines and growth factors in HIV-1 patients

		Cytokines/Chemokines, and Growth Factors Seen In HIV
Cytokine	**HIV-1**	**Reference**
ENA-78	↑↑	Clin Exp Immunol. 2002 Nov;130(2):279-8
GCSF	↑	Rev Mal Respir. 1997 Dec;14 Suppl 5:S142-51.
GRO	↑↑↑	Journal of Virology, July 2001, p. 5812-5822, Vol. 75, No. 13,
IL-1alpha	↑	AIDS. 2010 Mar 27;24(6):819-31
IL-1Beta	↑	mmunology. 2009 Sep;128(1 Suppl):e746-57
IL-2	↑	PLoS One. 2010 Oct 7;5(10):e13077
IL-4	↑	Biochem Biophys Res Commun. 2010 May 28;396(2):348-52
IL-5	↑	Cancer Causes Control. 2010 Aug;21(8):1323-33
IL-6	↑	AIDS Res Hum Retroviruses 1997, 13(9) 781-8, Cancer science vol 98, (9) 1288-1296
IL-7	↑	Eur Cytokine Netw. 2010 Sep 1;21(3):202-7
IL-8	↑↑	J Virol. 2010 Oct;84(20):10765-72
IL-10	↑	AIDS Res Ther. 2010 Oct 7;7(1):36
IL-12p40/p70	↑	J Leukoc Biol. 2010 Apr;87(4):645-53
IL-15	↑	Eur Cytokine Netw. 2010 Sep 1;21(3):219-21
IFN gamma	↑	AIDS Res Ther. 2010 Oct 7;7(1):36
MCP-1	↑	J Acquir Immune Defic Syndr. 2009 Dec 1;52(4):493-7
MCP-2	↑	FASEB J. 2010 Jul;24(7):2292-300
MIG	↓	J Clin Immunol 2010, 30(1) 90-8
MIP-1 Beta	↑	Science 1995, 270, 1811-15
RANTES	↑	Science 1995, 270, 1811-15
EGF	↑	Scand J Immunol 2007, 65(6) 549-54
Oncostatin M	↑	Science 1992, 255(5050) 1432-34
IGFBP-1	↑	Clin Chim Acta 2005, 361(1-2) 30-53
IGFBP-2	↑	Clin Chim Acta 2005, 361(1-2) 30-53
IGFBP-3	↓	Clin Chim Acta 2005, 361(1-2) 30-53
IL-16	↑	J Inf Dis, 1999, 179, 83-91
LIF	↑	AIDS 2006, 20(1)11-19
MIF	↑	Virology 2010, 399(1) 31-38
Osteprotegerin	↑	AIDS 2004, 18(5)475-83
TIMP-1	↑	J AIDS 2007, 46(3) 304-11

Table 1 shows a list of cytokines and chemokines expressed in HIV-1 infected patients. It is noted in the reports that a majority of cytokines and chemokines that have been associated with advancement of AIDS are increased.

HIV-1 infection increases the expression of most inflammatory cytokines and chemokines, and in our samples infection with other viruses, bacteria, and parasites may have initially enhanced the expression of these factors, and later maintained higher levels of some of the factors essential to tumorigenic cells.

Studies with cervical cancer have shown increased levels of IL-1β, IL-6, IL-8, IL-10, TNF-α, IFN-α, and β, that are believed to enhance pathogenicity associated with HPV in this cancer (Mindiola et al., 2008; Gasperini, Sakakibara, & Tosato, 2008). Similar findings have also been noted with Kaposi's sarcoma (Samanta, Iwakiri, & Takada, 2008), and Burkitt's lymphoma (Aggarwal, 2003). TNF has been shown to be involved in dysregulation of a number of major signaling pathways important for development of cancer when secreted into the circulation (Woodworth, McMullin, Iglesias, & Plowman, 1995). IL-1, IL-6, IL-8, and IL-18 can mediate different pathways that lead to cancer. For example IL-1 promotes cervical cancer growth (Klein et al., 1989), while IL-6 acts as a paracrine growth factor for non-Hodgkin's lymphoma (Klein et al., 1989).

Chemokines such as SDF-1α and MIP-3α are involved in cancer progression, including angiogenesis, inflammation, cell recruitment, and migration, and in recruitment and guiding of leukocytes to sites of inflammation (Charo & Ransohoff, 2006). It is noted that there is broad involvement of cytokines and chemokines in oncogenesis, and thus the stage, method and pathways used by these proteins in OSSN initiation and maintenance needs further analysis. Table 2 shows the cytokines, chemokines and growth factors reported to have been dysregulated in other cancers.

Table 2. Cytokine, chemokines and growth factors seen in cancer

Cytokine	Level Change	Cancer	Reference
ENA-78	↑	Renal	Acta Medica, 2008, 51(3), 185-90
GCSF	↑	Lung	Euro J Cardiothorc Surg, 2004, 26(4), 787-41
GM-CSF	↑	Colorectal	Int J Colorectal Dis, 2007, 22(1), 33-8
GRO	↑	Renal	Acta Medica 2008, 51(3), 185-90
IL-1alpha	↑	Prostate	Inflammation, 2008, 32(3), 202-10; Euro J Cancer, 1998, 34(6), 931-3
IL-1Beta	↑	Ovarion	Euro J Cancer, 1998, 34(6), 931-3
IL-2	↑	Breast	Int J Biol Markers, 2009, 24(3), 142-6
IL-4	↑	Bladder	Immunopharmacol Immunotoxicol, 2010
IL-5	↑	Bladder	Urol Oncol, 2009
IL-6	↑	Bladder	Immunopharmacol Immunotoxicol, 2010
IL-7	↑	Ovarion	Clin Cancer Res, 2007, 13(8), 2385-91
IL-8	↑	Ovarion	Clin Cancer Res, 2007, 13(8), 2385-91
IL-10	↑	Bladder	Immunopharmacol Immunotoxicol, 2010
IL-12p40/p70	↑	Hepatocellular Carcinoma	World Health J Gastroentorol, 2007, 13(32), 4345-9
IL-13	↑	Hodgkin's Disease	Blood 2001, 98(9), 2877-78
IL-15	↑	Head and Neck	J Larryngol Otol, 2007, 12(3), 246-52
IFN gamma	↑	Bladder	Urol Oncol, 2009
MCP-1	↓	acute myeloid leukemia	Neoplasma 2007, 54(4), 285-9
MCSF	↑	Colorectal	Int J Colorectal Dis, 2007, 22(1), 33-8
MDC (CCL-22)	↑	Hodgkin's lymphoma	Br J Haematol, 2008,140(5), 527-36
RANTES	↑	Malignant Thyroid	Proteomics Clin Appl, 2008, 2(12), 1575-85
SCF	↓	Colorectal	Digestive Diseases and Sciences
TARC(CCL-17)	↑	Hodgkin's lymphoma	Br J Haematol, 2008,140(5), 527-36

TGF-Beta1	↑	Pancreatic	Langenbecks Arch Surg, 2007, 392(3), 353-8
TNF-alpha	↑	Gastrointestinal Carcinoma	Int J Clin Pract, 2004, 58(6), 545-9
TNF-beta	↑	Gastrointestinal Carcinoma	Int J Clin Pract, 2004, 58(6), 545-9
EGF	↑	Gastrointestinal Carcinoma	Hepatogastroenterology, 2007, 54(76), 1049-52
Angiogenin	↑	Gastric Cancer	J Cancer Res Clin Oncol, 2003, 129(4), 239-44
Oncostatin M	↑	Colorectal Cancer	Anticancer Res, 2002, 22(23), 1045-52
Leptin	↑	Thyroid	Asian J Surg, 2009, 32(4), 216-23
TIMP-1	↑	Pancreatic Cancer	Pancreas, 2009, 38(6), 613-618
TIMP-2	↓	Bladder	Clin Biochem, 2007, 40(9-10), 640-4

Table 2 shows a list of cytokines and chemokines modulated in different cancers. From this table that shows a representative set of cytokines and chemokines expressed in different cancers, we note that in most of the cancers the factors are increased across the board indicating their influence on the pathologic outcome. The similarity of some of the cytokines and chemokines involved suggests that the pathways utilized by OSSN in its pathology may be the same with these other cancers.

The identification of the oncogenic viruses; HPV, KSHV, and EBV in OSSN and pterygia tissue samples suggest that these biological cofactors may be involved in the development of this malignancy in the HIV-1 population by partly eliciting the activities of cytokines, chemokines, and growth factors expressed or recruited by cancer cells required for proliferation (Simbiri et al., 2010). In this study we generated a cytokine and chemokine profile in OSSN and pterygia in HIV-1 infected patients in Botswana. We describe factors that these oncogenic proteins can elicit to maintain oncogenesis and the possible crosstalk involved. This study will thus provide a possible mechanistic view by which these tumor viruses elicit their oncogenicity in development of OSSN and pterygia pathogenesis.

2. Methods

2.1 Patient Samples

We enrolled HIV-1 infected patients with conjunctival lesions seen at Princess Marina Hospital, Gaborone, Botswana from April 11 2007 to April 14 2008 in the study (IRB #805049 and Ministry of Health , Botswana REF NO: PPME 13/18/1 Vol III 141). A total of 39 patients were enrolled. There were 13 males and 26 females. There were 30 OSSN cases of whom 19 were female and 11 males. There were 9 pterygia cases of whom 7 were female and 2 males. Thirty five sera were received, of which 27 were OSSN, 7 pterygia and 1 negative control without HIV or OSSN (Table 3). In this study based on the amount and condition of tissues and sera, we were only able to use 11 OSSN and 4 pterygium specimen. HIV-1 positive patients diagnosed using a HIV-ELISA (Abbott Laboratories, Hoofddorp, the Netherlands) with clinical features suggestive of OSSN or pterygium were enrolled the day before surgery into the study after signing consent form in English or Setswana. Tissue specimens obtained in the ophthalmology operating room were divided into two pieces by the ophthalmology surgeon - one piece was sent for histopathologic analysis and the other was immediately placed in tissue transport medium with sera and whole blood included for shipment to the University of Pennsylvania viral oncology laboratory. Histological confirmation of the specimen was obtained from the Botswana National Health Laboratory's histo- pathologist and University of Pennsylvania pathologist.

Table 3. Patient characteristics

	#	HPV		EBV		KSHV		CMV		HSV 1/2	
		#	%	#	%	#	%	#	%	#	%
Number of cases	39	24	(62)	30	(77)	25	(64)	23	(59)	25	(64)
Number of males	13	11	(84)	09	(69)	09	(69)	05	(38)	07	(53)
Number of females	26	13	(50)	21	(81)	16	(62)	17	(65)	18	(69)
Number of OSSN	30	20	(67)	23	(77)	07	(23)	16	(53)	19	(63)
Number of pterygia	09	04	(44)	07	(78)	05	(56)	06	(67)	06	(67)
Number of OSSN males	11	08	(73)	09	(82)	07	(64)	03	(27)	06	(55)
Number of OSSN females	19	12	(63)	15	(79)	13	(68)	13	(68)	12	(63)
Number of pterygia males	02	02	(100)	01	(50)	02	(100)	02	(100)	01	(50)
Number of pterygia females	07	02	(29)	06	(86)	04	(57)	04	(57)	05	(71)
Viral load <400	17	17	(100)	12	(71)	08	(47)	10	(59)	10	(59)
Viral load >400	07	07	(100)	06	(86)	06	(86)	04	(57)	04	(57)
Viral load ND	15	15	(100)	12	(80)	11	(73)	08	(53)	11	(73)
Antiretroviral prophylaxis	24	24	(100)	20	(100)	13	(54)	13	(54)	15	(63)
Antiretroviral ND	15	15	(100)	10	(67)	12	(80)	09	(60)	10	(67)
CD4 count <200	20	20	(100)	17	(85)	11	(55)	10	(50)	14	(70)
CD4 count >200	13	13	(100)	10	(77)	11	(85)	08	(62)	09	(69)
CD4 ND	06	06	(100)	03	(50)	03	(50)	03	(50)	01	(17)
Age 20-30	07	04	(57)	06	(86)	02	(29)	03	(43)	03	(43)
Age 31-40	17	08	(47)	13	(76)	13	(76)	10	(59)	11	(65)
Age 41-50	15	12	(80)	11	(73)	10	(67)	10	(67)	11	(73)

Table 3 shows characteristics of the cases in the study. It is observed that there were more OSSN than pterygia in the study, and more females than males. Because of antiretroviral application, most subjects had low viral load (<400) and higher CD4 counts (>200). However, the detection of herpes viruses was similar in OSSN and pterygia. The detection of the viruses was higher in those over 30 years old.

2.2 Cytokine Assay

Patient and control sera were diluted (1:4) in blocking buffer. Membranes coated with antibodies were blocked with blocking buffer for 30 minutes. The membranes (Ray Biotech, Inc. Norcross, GA) were incubated at 4^0C overnight with the sera samples. The membranes were washed 3 times using 1X wash buffer I, followed by 1 time with wash buffer II (Ray Biotech, Inc. Norcross, GA). The membranes were incubated with Biotin-conjugated anti-cytokine (Ray Biotech, Inc. Norcross, GA) for 2 hours at room temperature and washed with buffers I and II and incubated with Alexa 800 conjugated Streptavidin (Invitrogen, Carlsbad, CA) for 2 hours. The membranes were washed with buffers I and II and detection done immediately using Odyssey V3.0 (Lincoln, Nebraska).

2.3 Quantitative Real Time-PCR

Tissues from 11 OSSN, 4 pterygia and 4 negative conjunctival control were deparaffinized in xylene (2 times), and dehydrated in absolute alcohol (3 times). RNA was extracted and cDNA prepared accordingly (Applied Biosystems, Foster City, CA). Expressions of the different cytokines were determined by RT-PCR using a Step One Plus Real Time PCR System (Applied Biosystems, Foster City, CA). The cDNA was amplified using Power SYBR green PCR master mix (Applied Biosystems, Foster City, CA), 1 μM each primer(Table 4), and 1-3 μl (25-75ng) of the cDNA product in a total volume of 20 μl. Thirty-five cycles of PCR (1 cycle consisting of 1 min at 94^0C, 30 s at 48^0C to 62^0C (depending on the primer), and 40 s at 72^0C), followed by 60 s at 72^0C. Relative quantitation was calculated by the ΔΔCt method (Cai, Verma, Choi, Ma & Robertson, 2010). All experiments were performed in triplicate.

Table 4. Specific primer sequences used in RT-PCR

	Forward Primer	Reverse primer
IL-4	5′- GCCTGGCGGGCTTGAATTCCTGT -3′	5′- TCAGCTCGAACACTTTGAAT -3′
IL-6	5’- TGCCTGGTGAAAATCATCACTGGTC - 3’	5’- GTGGTTATTGCATCTAGATTCT -3’
IL-8	5’- CAATAATTTCTGTGTT -3’	5’- CAG'TTTTGCCAAGGAGTGCT -3’
IL-10	5’- TGTCATCGATTTCTTCCCTGTGA -3’	5’TCTCTTGGAGCTTATTAAAGGC -3’
IL-13	5’- AGAAGGCTCCGCTCTGCAAT - 3’	5’- AAAACTGCCCAGCTGAGACCTTG-3’
CD4	5’- CACCGAAGGCGCCAAGCAGAGC -3’	5’- TTCTGAAACCGGTGAGGA-3’
CD8	5’- GCAGTGCACACGAG-3’	5’-GATTTGACCACAGGCCG-3’
NF-κB	5’- GGTATAGCTTCCCACACTAT-3’	5’-TAGATTCAGTGTCCATGGTTC-3’
TGF-β	5’- AGCTCCACGGAGAAGAACTGC-3’	5’-CAGGGCCAGGACCTTGCT-3’
MCP-1	5’ -ATGAAAGTCTCTGCCGCCCTT -3’	5’- GGTCTTGAAGATCACAGCTTCT-3’
TNF-α	5’- CACCCATGTGCTCCTCACCCA-3’	5’- AGATAGATGGGCTCATAC-3’
TNFSF13B	5’- TACGCCATGGGACATCTAATTCAGA-3’	5’-GTTTCAGGCATATTTGAATAC-3’
VEGF	5’- ACATCACCCATCCCACTC-3’	5’- ACATCACCCATCCCACTC-3’
GAPDH	5′-TGCACCACCAACTGCTTAG-3′	5′- GATGCAGGGATGATGTTC-3′

Table 4 shows the list of specific primer sequences used in the quantitative Real Time-PCR experiments. Thirty-five cycles of PCR (1 cycle consisting of 1 min at 94^{0}C, 30 s at 48^{0}C to 62^{0}C, depending on the primer, and 40 s at 72^{0}C), followed by 60 s at 72^{0}C.

3. Results

3.1 Expression of Inflammatory Cytokines, Chemokines and Growth Factors were Modulated in OSSN and Pterygia

Potential dysregulation of cytokines, chemokines and growth factors and their contribution to the pathogenesis of OSSN and pterygia was analyzed using serum samples obtained from 2 OSSN (#19 and #20), and 2 pterygia (#15 and #16) patients as well as a negative control subject from the same area for analysis. Several cytokines and chemokines were expressed by OSSN, pterygia, and the negative control subject. The results of cytokine array assays showed the modulation of expression of several inflammatory factors. Notably, the levels of cytokines and chemokines from OSSN and pterygia subjects were consistently lower than the control subject. Furthermore, the levels of these cytokines and chemokines were not significantly different between pterygia and OSSN, except for VEGF, TNF-β2, Angiogenin, and Gro which were high in pterygia and low in OSSN (Figure 1).

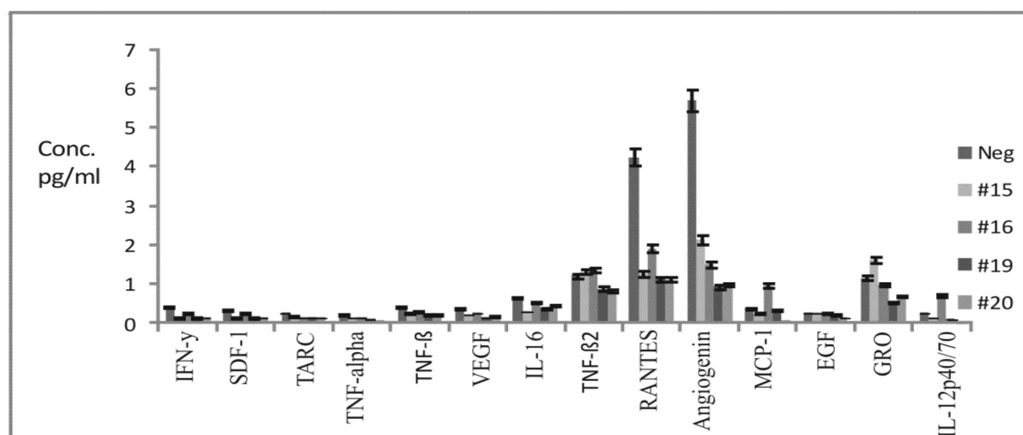

Figure1. Low expression levels for cytokines, chemokines and growth factors in serum of OSSN and pterygia patients

Figure 1 shows cytokines and chemokines expressed in sera of OSSN and pterygia patients. In the assay we used 1ml of 2-fold to 5-fold diluted sera. Patient and control sera were diluted (1:4) in blocking buffer. Membranes coated with antibodies were blocked with blocking buffer for 30 minutes. The membranes (Ray Biotech, Inc. Norcross, GA) were incubated at 4^0C overnight with the sera samples. The membranes were washed 3 times using 1X wash buffer I, followed by 1 time wash buffer II. The membranes were incubated with Biotin-conjugated anti cytokine (Ray Biotech, Inc. Norcross, GA) for 2 hours at room temperature. The membranes were again washed with buffers I and II and incubated with Alexa 800 conjugated streptavidin (Invitrogen, Carlsbad, CA) for 2 hours. The membranes were washed with buffers I and II and detection done immediately using Odyssey V3.0 (Lincoln, Nebraska). TNF-β2, RANTES, and Angiogenin were highly expressed in the control and patient sera, IL-16, VEGF, GRO, and TNF-β were higher in all cases. The negative control expressed higher levels of all the cytokines and chemokines than the patients.

The levels of pro-inflammatory cytokines such as GRO, IFN- , IL-16, RANTES and IL-12p40/p70 detected in most cases were lower than the negative patient sera (Figure 1). These factors are normally secreted and increased to higher levels in most infections and cancer than what we observed with our samples. We made the same observation with pro-inflammatory factors TNF-α, and TNF-β which were secreted in low levels and did not differ between OSSN and pterygia. We also noted the secretion of growth factors VEGF, TNF-β2, Angiogenin, and EGF were to appreciable levels (Figure 1). The assay also showed increased levels of Chemotactic factors GRO, RANTES, and MCP-1 (Figure 1).

3.2 The Level of CD4 and CD8 Expression Was Low in OSSN and Pterygia Tissue Samples

All patients included in the study were HIV-1 infected and on Antiretroviral therapy (ARTs), with therapy starting at different stages, hence some patients still had higher viral loads and low CD4 counts while the rest had low viral loads and appreciable CD4 counts. There were some patients whose CD4 and viral loads were not available. From the few subjects that we had, pterygium cases tended to have low viral loads and higher CD4 counts than OSSN patients, an indication of a slightly healthier status than OSSN (Table 5). However, when the expression of CD4 and CD8 T cells by quantitative RT-PCR was performed on tissue samples, we noted that signals for CD4 expression was drastically lower in most of these tumors with some having little or negligible detection. However, CD8 expression was similar to control for two samples but significantly higher than CD4 for most (Figure 2 A and B). Thus, CD8 expression was more prominent in the OSSN tumors compared to CD4 in both OSSN and pterygia tissues.

Table 5. CD4 counts, viral loads of the subjects used and diagnosis of patients in study cohort

Case #	Diagnosis	CD4 count	HIV Viral load
6	OSSN	174	120,000
13	OSSN	UNK	<400
17	Pterygium	491	UNK
21	OSSN	UNK	Not done
23	OSSN	220	<400
25	Pterygium	293	<400
26	Pterygium	546	<400
27	Pterygium	113	UNK
29	OSSN	192	UNK
31	OSSN	62	17,000
32	OSSN	107	<400
36	OSSN	38	200,000
37	OSSN	UNK	UNK
38	OSSN	31	UNK
39	OSSN	121	UNK

Table 5 shows the diagnosis, CD4 counts, and HIV-1 viral load of patients whose RNA was extracted from slides for cDNA analysis of cytokine expression. From the small sample size, we note that pterygia cases had higher

levels of CD4 counts and lower viral loads than OSSN cases amongst those whose complete data was available.

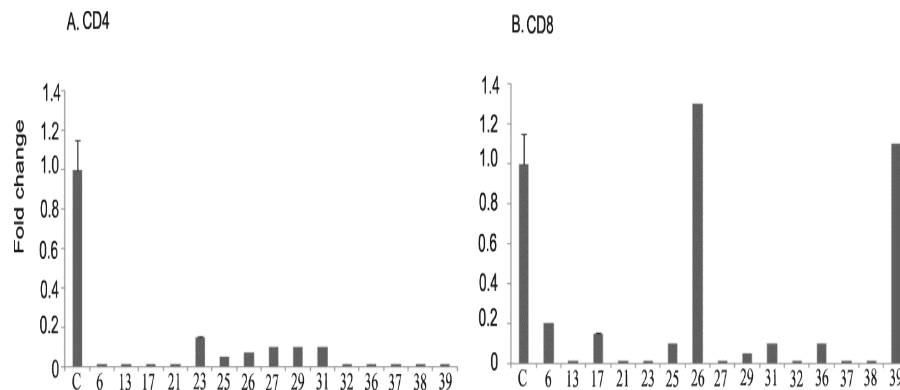

Figure 2. Analysis of CD4 and CD8 transcripts levels in OSSN and pterygia

Using primers for several cytokines and chemokines that have been observed to be expressed at higher levels in some cancers and HIV-1 infected patients we prepared cDNA from RNA extracted from patient slides. The analysis was done on all samples listed in table 4. Using AB(Applied Biosystems, Foster City , CA) 10 µl 2X RT buffer, 1µ of 20X RT enzyme mix, 2µg of RNA, and 9 µl of DEPC water to make a volume of 20µl. The samples were briefly centrifuged and reverse transcription performed at 37^0C for 6 minutes, 95^0C for 5 minutes and 4^0C infinity. The cDNA was amplified on the Step One Plus Real Time PCR System using Power SYBR green PCR master mix (Applied Biosystems, Foster City, CA), 2µl of 1 µM primer, 3µl of SYBR Green, and 1-3 µl (25-75ng) of the cDNA product in a total volume of 20 µl. Thirty-five cycles of PCR (1 cycle consisting of 1 min at 94^0C, 30 s at 48 to 62^0C (depending on the primer), and 40 s at 72^0C), followed by 60 s at 72^0C. Ct values for the relative quantitation were calculated by the ΔΔCt method. Transcript quantification results were again normalized against GAPDH DNA content. The experiments were performed in triplicate.

Representative factors expressed at low levels by OSSN and pterygia samples from Botswana were analyzed. CD4 (2A) and CD8 (2B) expression were expected to be low in these tissues since there are normally few to none T cells expressing CD4 and CD8 in conjunctival region of the eye. Higher levels of CD8 seen in # 26 and #39 could be a result of infiltration of the eye by cytotoxic CD8 attempting to clear viral infection. Control value is an average of 4 conjunctival control samples.

3.3 Transcript Levels for the Pro-Inflammatory Factors were Modulated in OSSN and Pterygia

We performed quantitative RT-PCR on representative cases of OSSN and pterygia using specific primers (Table 4) designed for cytokines, chemokines and other markers. We identified IL-4 (Figure 3A), a factor shown to be involved in proliferation and Th cell differentiation and able to block MIP-1α, IL-1, IL-6, IL-8, and TNF-α (Mosmann, Cherwinski, Bond, Giedlin, & Coffman, 1986), Tumor necrosis factor (TNF) a monocyte-derived cytotoxin that has been implicated in tumor regression, septic shock and cachexia (Kriegler, Perez, DeFay, Albert, & Lu, 1988), TNF-13B an important member of the TNF superfamily known to regulate B cell proliferation and differentiation and Ig production (Salzer, Jennings, & Grimbacher, 2007). The majority of the samples tested expressed TNF (Figure 3B) and TNF-13B (Figure 3C). Of interest were the high fold increases of TNF and TNF-13B, in both #13 and #29 patient samples although there were some samples where the levels were lower and 3 where little or no signal for TNF-13B was detected (Figure 3B and C). We also observed expression of TNF in samples #17, 21, and 23, but the same samples did not have similar TNF-13B signals (Figure 3B and C), indicating that the TNF expressed was of a different superfamily. This suggests that even though the patients could have been exposed to the same pathogens, their response to the pathogens will vary based on multiple factors such as the loads of the pathogens and level of immunosuppression.

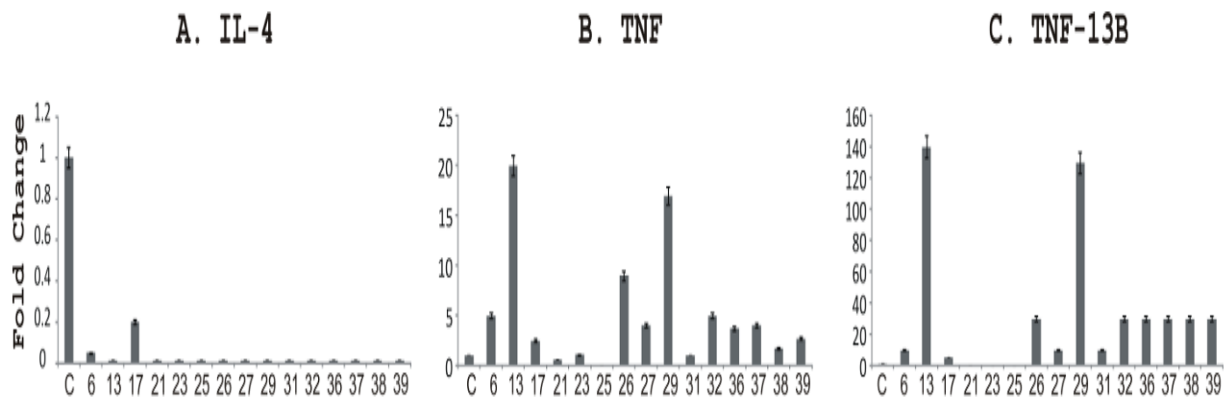

Figure 3A, 3B and 3C. Determination of pro-inflammatory cytokine levels in OSSN and pterygia

Surprisingly pro-inflammatory cytokines IL-4(3A) which is normally expressed at higher levels in HIV-1 infected and cancer patients was low, possibly a result of multiple immunosuppression by HIV-1, OSSN/pterygia, and other infections, including opportunistic infections. Yet TNF (3B) and TNF-13B (3C) cytokines expressed in most infections and cancer was expressed to appreciable levels by #6, #13, #17, #26, #27, #29, #s31-#39. Control value is an average of 4 conjunctival control samples.

3.4 Anti-Inflammatory Cytokines are Expressed in OSSN and Pterygia

Using specific primers we demonstrated the expression of the anti-inflammatory factors IL-10 (Figure 3D), a protein that inhibits the synthesis of other cytokines like IFN- , IL-2, IL-3, TNF, and GM-CSF (Zdanov, Schalk-Hihi, Gustchina, Tsang, Weatherbee, et al.1995; Alonso, Pontiggia, Medenica, & Rizzo, 1997). No detectable increase was observed with IL-13 in the patient samples although the control showed nice signal (Figure 3E). IL-13 which down-regulates the production of TNF, IL-1, IL-8, and MIP-1α by monocytes (de Waal Malefyt, Figdor, Huijbens, Mohan-Peterson, Bennett et al.1993) not expressed in the tissue samples.

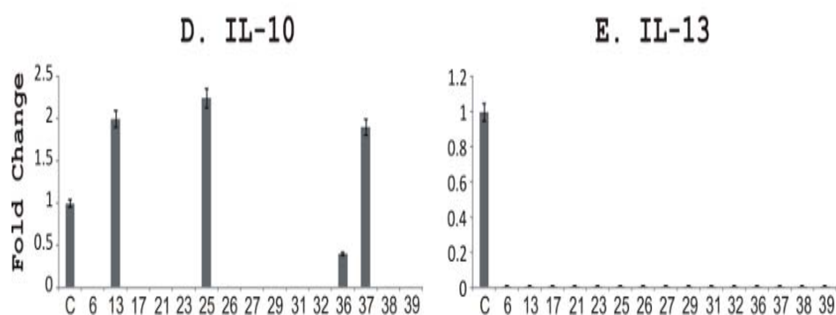

Figure 3D and 3E. Low expression of IL-10 and IL-13 in OSSN and pterygia

All cDNA used in the analysis were extracted from the listed samples in Table 4. Representative anti-inflammatory cytokines IL-10 (3D) and IL-13(3E) were expressed at low levels by OSSN and pterygia samples from Botswana are shown, with IL-10 expressed by only #13, #25, #36, and #37, while none of the samples expressed IL-13 to appreciable levels. Control value is an average of 4 conjunctival control samples.

3.5 Growth Factors were Expressed by OSSN and Pterygia

Quantitative RT-PCR was used to determine the expression of VEGF which may affect the physiological and pathological outcome in angiogenesis including cancer (Clendenen et al., 2011). We showed a relatively higher fold increase in signal with patient #s 6, 13, 29, 32, 36, 37, and 39 showing particularly high levels (Figure 3F). TGF-β which can display both pro-inflammatory and anti-inflammatory properties depending on context (Dourado, Martinez-Maza, Kishimoto, Suzuki, & Detels, 1997) had an appreciable increase in most samples

especially in #s 21 and 27 representing OSSN as well as pterygia (Figure 3G). IL-6 which is involved in differentiation of B-cells into Ig-secreting cells has both pro-inflammatory and anti-inflammatory properties (Barton, 1997). Only two of the samples, #21 and #29 showed increased signals for IL-6 above that for the control (3H).

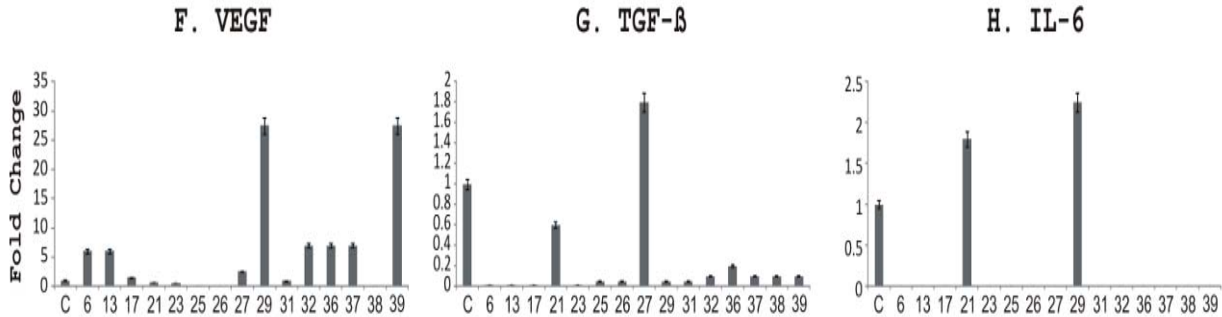

Figure 3F, 3G and 3H. Most OSSN and pterygia samples express VEGF and TGF-β, except for IL-6

Representative growth factors VEGF, TGF-β, and IL-6 are shown. VEGF (3F) which has been shown to be expressed at high levels in HIV-1 infection and cancer was consistent with expectation as it was expressed in most of the samples. TGF-β (3G) was not as robustly expressed as VEGF in these samples. IL-6 (3H) which is normally expressed at higher levels in HIV-1 and cancer was expressed at higher levels by 2 samples. Control value is an average of 4 conjunctival control samples.

3.6 Chemotactic Factors were Rarely Expressed by OSSN and Pterygia

The Chemotactic cytokines IL-8, an angiogenic chemokine with roles in development and progression of many cancers (Chien, Yeh, Li, Wei, Chang, et al. 2011) was expressed in two samples #21 and #25 (Figure 3I). MCP-1 (CCL2), a member of the CC chemokine family that regulates monocyte migration by enhancing transit from the bone marrow into the circulation or from circulation to the site of inflammation (Tsou, Peters, Si, Slaymaker, Aslanian, et al. 2007), was expressed by #21 (Figure 3J). The majority of the samples had little or no detectable signals for these two chemotactic factors. We did not expect to have fewer samples express low levels of these chemotactic cytokines that are usually associated with cancer and infections despite a majority of the patients being immunosuppressed.

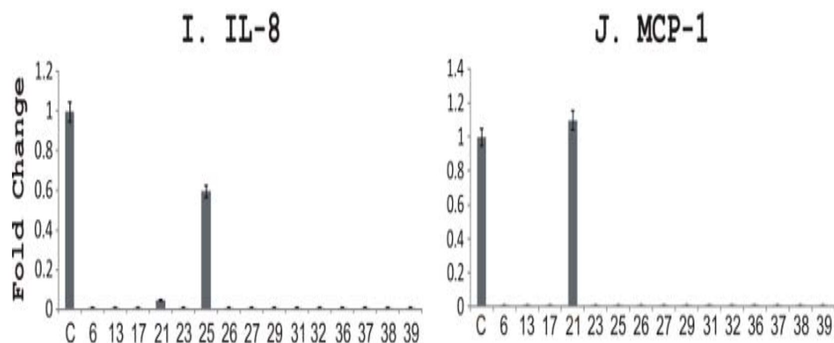

Figure 3I and 3J. OSSN and pterygia poorly express chemotactic factors

It was noted that most samples did not express chemotactic factors as shown here by IL-8 (3I) and MCP-1(3J). Only one tissue sample expressed each of the factors from the panel tested. Control value is an average of 4 conjunctival control samples.

Of interest was the observation that the cytokines and growth factors observed in cytokine assay using serum were also seen in RT-PCR experiments using RNA extracted from tissues, an indication that the sera results were not some global response to infection, but could have been more specific to pathogens and cancer in the tissues (Table 6).

Table 6. Representative Factors detected in sera and tissues of patients

Chemokine/cytokine	Sera	Tissue
Interferon- ɤ	+	ND
SDF-1	+	ND
TARC	+	ND
TNF-α	+	+
TNF-β	+	+
TNF-B13	+	+
VEGF	+	+
RANTES	+	ND
TGF-β	low	+
Angiogenin	+	ND
MCP-1	+	+
MIP-1	+	ND
EGF	+	ND
GRO	+	ND
IL-12p40/70	+	low
IL-16	+	ND
IL-13	low	low
IL-10	low	+
IL-8	low	+
IL-6	low	+
IL-4	low	+

ND-Not Determined

4. Discussion

It has been reported that HIV-1 infections initially induce anti-viral CTL response, but that with enhanced virus replication the viral "allergen-like domains" in the viral structural proteins that are presented by HLA class II molecules on dendritic cells to Th_2 cells induce the synthesis of IL-4, IL-5, and IL-13 (Becker, 2004). The increase in levels of the Th_2 cytokine IL-4 inhibits the ability of Th1 cells to synthesize IL-2, IL-12, and IFN-γ as well as antiviral CTL activity and CTL precursors (Becker, 2004). The progression of HIV-1 infection and other opportunistic infections leads to AIDS and a complete failure of the immune system which can resemble an untreated allergy (Becker, 2004), that eventually predisposes patients to cancers.

To our knowledge this is the first study to assess the expression of different cytokines and chemokines in OSSN and pterygia. The results of our study show that in OSSN and pterygia there is a marked suppression of both Th_1 and Th_2 type cytokines. Th_1 cells that elevate cell mediated immunity in response to intracellular pathogens are inhibited by Th_2 cells that favor humoral immunity in response to extracellular pathogens. Unlike some cancer cases in which the imbalance in Th_1/Th_2 takes place with Th_2 predominating (Becker, 2004; Satyam, Singh, Badjatia, Seth, & Sharma, 2009), the samples from immunocompromised patients which involves both intracellular and extracellular infection, the mutual inhibitory effect would likely be constitutive leading to suppression of both arms of the immune system. Decreased amounts of Th_1 cytokines indicate that proliferation and persistence of tumor cells may be due to the immunocompromised state of the patients which may have a vital role in inhibiting antitumor activities. In most advanced cancer cases, IFN-γ and other Th_1 cytokines drop as our findings show (Satyam et al., 2009). Alonso et al (1997) in a study of adults with AIDS observed minimal elevations for IL-2, IL-6, IL-8, IL-12, IFN-α, IL-6SR in PBMCs compared to controls, and the level of RANTES was lower than normal controls. Cytokine elevations in AIDS patients are reflective of chronic viral infection, which in our cohort included HIV-1 and herpes viruses (Nicol et al., 2005; Fernandes et al., 2005). In the samples we analyzed there was no significant difference between OSSN and pterygia and both cytokines and chemokines were expressed similarly. This may indicate that pterygium from HIV-1 infected Botswana patients may in fact be a precursor of OSSN as suggested in our previous paper, a hypothesis that will warrant verification by following a cohort of pterygia patients to observe if they develop OSSN overtime (Simbiri et al.,

2010). In the cytokine and chemokine array screen it was observed that the negative control responded stronger than the cases in all cytokines and chemokines analyzed, and that the response from pterygia was slightly higher than OSSN. The stronger response from the negative control may have been due to an infection and a response from an immunocompetent individual which was stronger. However, the expression of the cytokines was mixed in other cancers such as cervical cancer where cytokine expression is dependent on stage and HPV types, which may apply to OSSN (Fernandes et al., 2005; Pardo-Govea et al., 2005; Rajappa, Saxena & Sharma, 2007).

RT-PCR showed the expression of inflammatory cytokines such as TGF-β, TNF, and TNF-13B. NF-KB which is activated by cytokines and is a transcriptional factor that regulates a battery of genes that are critical to innate and adaptive immunity, cell proliferation, inflammation, and tumor development was significantly increased in our samples (not shown) (Ma, Becker Buscaglia, Barker, & Li, 2011). VEGF is an important angiogenic factor, with significant effects on tumor angiogenesis and the only highly expressed angiogenic factor in our samples (3F). Some studies have demonstrated that VEGF could be a prognostic factor, independent even from microvascular density, which is increased by its expression (Baderca, Alexa, Lighezan, Izvernariu, & Raica, 2011). TGF-β, depending on the microenvironment can be proinflammatory by being a potent chemoattractant for neutrophils and promoting inflammation (Mantel & Schmidt-Weber, 2011). It also induces differentiation into the anti-inflammatory Treg cells and the proinflammatory Th_{17} and Th_9 cells but inhibits Th_{22} differentiation (Mantel & Schmidt-Weber, 2011). In infections, it protects against collateral damage caused by the immune system, but it also promotes immune evasion from chronic infections. In autoimmune diseases, its dysfunction leads to the loss of tolerance to self-antigens (Mantel & Schmidt-Weber, 2011). In cancer, TGF-β is a potent inhibitor of cell proliferation and acts as a tumor suppressor at the beginning of tumorigenesis. However, once the cells become resistant to TGF-β, it sustains tumor growth and metastasis by promoting immune evasion and angiogenesis (Mantel & Schmidt-Weber, 2011). Chemokines like MCP-1, and other cytokines such as IL-4, IL-6, IL-8, IL-10, and IL-13 were not elicited to appreciable levels in our study. Other cancers show increased levels of these cytokines (Clendenen et al., 2011; Ma et al., 2011; Baderca et al., 2011). The lower expression of these cytokines in our samples could be due to tissue specificity and multiple viral and other infections that increase immunosuppression seen in our patient cohort.

In a study by Dourado et al. (1997), they reported prevalent elevation of IL-6 among men with AIDS and opportunistic infections than those with AIDS and Kaposi's sarcoma. They noted that the high prevalence of IL-6 among controls could be explained by association of higher levels of IL-6 and lower levels of CD4 T cell number. In our samples though pterygia cases had higher CD4 counts and lower HIV-1 viral load than OSSN, there was no difference in expression of IL-6 and indeed other cytokines between the two groups, though due to sample size a larger study is warranted.

Clerici et al. showed that immunoinhibitory Th_1 cytokines IL-4 and IL-10 capable of stimulating tumor growth are linked with cervical cancer and CIN grade III compared to early CIN and healthy controls (Clerici et al., 1997). Nicol et al looking at effects of HIV-1 co-infection in HPV infected women observed an increased number of cells that expressed IL-6, IFN-α, IFN- , and TNF-α in HPV infection, while co-infection led to increased number of cells expressing IL-4, IL-10, and IL-8 (Nicol et al., 2005). Co-infection with HIV-1 was also associated with higher numbers of CD8. Similar to some of our findings Chopra et al (1998) observed increased serum levels of Angiogenin, IL-2, IL-6, IL-7, IL-8, IL-10, b-FGF, TNF-α, TGF-β, TNF-β, and GM-CSF during different stages of cervical cancer (Chopra, Dinh, & Hannigan, 1998). It is to be assumed that the expression of the cytokines would vary in the different stages and grades of pterygia and OSSN depending on HIV-1 infection and other infections. To elucidate this would require a larger study with a larger cohort of controls. Thus without proper controls of subjects with HIV+/-OSSN and HIV-/+OSSN it is difficult to decipher whether it is HIV-1, OSSN, or both contributing equally to the immunosuppression affecting release of cytokines and chemokines. The data however, shows that both are involved to different levels which may vary with the stage of disease and infectious agents acting actively or passively in the individual patient, and other epigenetic and extraneous factors.

Kwon and Kaufmann (2010) reported that genetic polymorphisms in the IL-10 gene promoter that lead to decreased IL-10 expression have been associated with more rapid disease progression in late stages of HIV infection that suggest the anti-inflammatory effects of IL-10 may be protective in the setting of chronic immune activation. Further, Furler and Uittenbogaart (2010) argue that cytokine and biomarker expression in HIV-1 infection results from the combined effect of intracellular signaling pathways orchestrated by kinases like p38 and ERK, and that p38, ERK and Mitogen-Activated Protein Kinase (MAPK) pathways govern the regulation of cytokines (IL-2, IL-10, and TNF-α) as well as biomarkers (PD-1, Fas/FasL, among others) that are skewed in chronic HIV infection. Additionally, HIV utilizes the p38 and ERK pathways to produce new virions to deplete

$CD4^+$ T cells from the host's immune system that would contribute to some of the cancers seen in HIV-1 infected patients.

Noguchi et al. (2007) showed that EBER can activate NF-kB and IRF3 pathways. EBER-mediated signaling has been associated with cytokine expressions in EBV-infected cells. The activation of NF-kB and AP-1 by viral antigens leads to expression of cellular IL-6, IL-8, bFGF, and VEGF, which contribute to host cell survival and malignancy. Over 75% of our cohort tested positive for the ubiquitous EBV by PCR in tissue (Simbiri et al., 2010). Hence the effect of EBER on the expression of these cytokines, plus other viral proteins calls for a detailed analysis.

The profile of cytokines, chemokines and growth factors , though different from patient to patient in our OSSN and pterygia HIV-1 patients suggests that multiple viral and other infectious agents exposure may lead to dysregulated expression of proinflammatory factors that have been associated with onset and maintenance of cancer. However, the varied levels between patients could be attributed to stage of the disease, level of immunocompetence, and the presence of other infectious and opportunistic agents. Of significance was the detection of similar cytokines, chemokines, and growth factors in both the sera and tissues (Table 6).

5. Conclusion

This study investigating the expression of cytokines and growth factors in OSSN and pterygia in HIV-1 patients suggest that multiple viral and other infectious agents known to modulate expression of cytokines and other factors may contribute to the development of OSSN and pterygia. The interaction between different pathogenic agents and the dysregulatory activities of these factors in immunocompromised individuals will contribute to cell survival and proliferation of the infected cells. Clearly, further study is warranted to elucidate the molecular processes and pathways involved in pathogenesis of OSSN and pterygia in HIV-1 population.

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Irinotecan and Capecitabine (CAPIRI) Plus Bevacizumab in First-Line Treatment of Metastatic Colorectal Cancer

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Abstract

The purpose of this prospective study was to assess the efficacy and safety of bevacizumab in combination with reduced doses of irinotecan plus capecitabine (CAPIRI regimen), in patients with metastatic colorectal cancer (mCRC), as first-line chemotherapy. A cohort of 120 mCRC consecutive patients was included. The overall response rate was 63.3% (76 patients; 95% confidence interval [CI], 53.97%-71.77%). Median time to progression and overall survival were 15 months (range: 2-49 months; 95% CI: 13.00, 17.00 months) and 22.5 months (range: 4-54 months; 95% CI: 21.00, 27.00 months), respectively. The one year survival rate was 81.5%. CAPIRI-related grade 3/4 adverse events included alopecia (29.2%) and diarrhoea (16.7%), which were manageable. Bevacizumab-related grade 3 hypertension was reported in 2 patients. One patient died due to treatment related adverse event, which was no bevacizumab-related. In conclusion, combination of bevacizumab plus CAPIRI is a feasible treatment which provides a clinical benefit as first-line treatment in chemonaïve patients with mCRC.

Keywords: irinotecan, capecitabine, bevacizumab, colorectal neoplasms, neoplasm metastases

1. Introduction

Approximately 60% of patients with colorectal cancer need chemotherapy to treat their metastatic disease. Chemotherapy was shown to increase the quality of life, time to disease progression (TTP) and overall survival (OS) of patients with metastatic colorectal cancer (mCRC). The introduction in recent years of new chemotherapeutic treatments (e.g. capecitabine, irinotecan, or oxaliplatin) or new regimens with monoclonal antibodies that inhibit specific molecular targets (e.g. bevacizumab, cetuximab, or panitumumab) have substantially improved the efficacy (Colucci et al., 2005; de Gramont. et al., 2000; Hurwitz et al., 2004; Simpson et al., 2003; Tournigand et al., 2004).

Capecitabine offers the advantage of continuous exposure to 5-FU without requiring central venous access (Cassidy et al., 2002; Van Cutsem et al., 2004) and, therefore, it is more convenient to administer, requires less hospitalization and decreases the utilization of medical resources (Payne, 1992). Capecitabine was developed, in combination with either oxaliplatin or irinotecan in a 3 week schedule, instead of 5-FU infusion, as an interesting alternative due to the practicality of the treatment. Moreover, the use of capecitabine instead of 5-FU, either with irinotecan or oxaliplatin, confirmed the activity and efficacy of the drug (Cassidy et al., 2004; Koopman et al., 2007). However, to date no study has shown which regimen (oxaliplatin based or irinotecan-based) is the best in the first and successive lines of treatment. In fact, two studies by Goldberg et al. (2004) and Tournigand et al. (2004) comparing oxaliplatin and irinotecan regimens found no differences in their survival rate and safety profiles. Likewise, studies comparing oxaliplatin and irinotecan regimens in combination with capecitabine were very scarce, as capecitabine doses used in initial comparative studies resulted in an unacceptable level of toxicity (Fuchs et al., 2008). Nevertheless, results from a phase II study by Grothey et al. found minimal differences in survival between capecitabine+oxaliplatin and capecitabine+irinotecan regimens (Schmoll et al., 2006).

In addition, it is important to avoid drugs with cumulative toxicity which can limit their benefits. For oxaliplatin-based regimens, severe peripheral neuropathy led to treatment discontinuation in more than 20% of patients after six months of treatment (Gamelin et al., 2002; Grothey, 2003; Krishnan et al., 2005). Moreover, 5% of the sensitive neuropathies were permanent or persisted for more than two years (de Gramont et al., 2000;

Giacchetti et al., 2000; Hospers et al., 2006). On the other hand, the major adverse events (AEs) associated with irinotecan are diarrhoea and neutropenia, being not cumulative and allowing for continued treatment until disease progression.

New regimens with targeted drugs (e.g. bevacizumab) have shown to prolong the TTP as well. Bevacizumab, a humanized monoclonal antibody against vascular endothelial growth factor, combined with fluoropyrimidine based chemotherapy is one of the standard regimens in first line treatment of mCRC and has demonstrated a consistent benefit in several studies (Hurwitz et al., 2004; Macedo et al., 2012).

To gain further information on the efficacy and safety of bevacizumab in combination with reduced doses of capecitabine plus irinotecan (CAPIRI) in a 3-week schedule we decided to prospectively collect the data on this combination from a cohort of mCRC patients attending to our centre. The more favourable cumulative toxicity profile and convenience, made us prefer the low dose CAPIRI treatment in combination with bevacizumab over an oxaliplatin-based regimen. Reduced doses of irinotecan and capecitabine were chosen to increase compliance with the chemotherapeutic regimen while maintaining dose intensity within the activity range for these drugs (Kim et al., 2005). The low dose CAPIRI regimen is extensively used in our unit with acceptable tolerability, based on a previous phase I-II study with irinotecan in second line set conducted in our department (Vieitez et al., 2003). Here, we report the results of this non interventional, single-centre study.

2. Method

2.1 Study Design

The study was performed after obtaining approval from the local Institutional Review Board committee and in accordance with the Declaration of Helsinki, the Good Clinical Practices, and local ethical and legal requirements. The study was performed under standard clinical practice conditions in mCRC patients treated at the University Central Asturias Hospital in Spain. Before inclusion, all patients were fully informed about the study and all gave their written consent.

2.2 Study Population

Patients with histologically confirmed unresectable mCRC, with measurable lesions according to Response Evaluation Criteria in Solid Tumours (RECIST) (Therasse et al., 2000) by computed tomography (CT) scan; aged ≥18 years and Karfnosky performance status (PS) ≥60% were included. Prior chemotherapy for advanced disease was not permitted, but adjuvant or neoadjuvant chemotherapy was allowed providing it was completed at least 12 weeks before inclusion. Prior radiotherapy was permitted if there were measurable lesions outside the radiation field at inclusion. Prior radiotherapy/major surgery must have been completed at least 6 weeks before inclusion. In the absence of symptoms (bleeding, obstruction, and perforation) patients without primary tumour resection or with ascites were eligible.

2.3 Methods

Patients received CAPIRI + bevacizumab treatment, which consisted of a 90 minutes IV infusion of irinotecan 240 mg/m^2 on day 1 plus oral capecitabine 850 mg/m^2 twice daily for 2 weeks plus bevacizumab 7.5 mg/Kg IV on day 1 in a 3-week cycle. Delays or dose reductions for capecitabine (up to 40%) or irinotecan (up to 20%) were permitted according to the tolerance on previous cycle. Chemotherapy was continued until progression, death, unacceptable toxicity or refusal, or lost to follow-up.

The following information was collected from medical records at baseline visit: patients' medical history, Karfnosky PS assessment, and significant findings in blood and urinary tests (including proteinuria and CEA measurement levels). Data from tumour assessments performed were collected from baseline visit and, thereafter, every 2-3 months. The centre's standard clinical practice for tumour assessments includes CT evaluation of the chest, abdomen, and pelvis. Tumour responses were scored according to RECIST v1.0 criteria recommendations (Therasse et al., 2000). The safety was evaluated for all patients receiving at least one dose of the treatment cycle and graded according the National Cancer Institute Common Toxicity criteria (NCI CTCAE) version 2.

Complete liver metastatic resection with curative intention was attempted in patients who were deemed resectable after chemotherapy.

2.4 Statistical Analyses

The primary endpoint of this study was the overall response rate (ORR) assessment; i.e. the percentage of responders (complete response [CR] + partial response [PR]) to treatment with CAPIRI + bevacizumab. The statistical software "Ene-2.0" (Badiella Busquets & Pedromingo, 2010) was used to determine the needed

sample size. Assuming a minimum efficacy of 44.8% (the ORR reported by Hurtwitz et al (Hurwitz et al., 2004) for the IFL + bevacizumab group), a precision of 7.5%, unilateral $\alpha = 0.05$, and $\beta = 0.20$, 119 patients were required. Secondary endpoints included the evaluation of TTP, OS, and the safety profile.

Efficacy analyses included all treated patients. Safety analyses included all patients who received at least one dose of the treatment cycle. TTP was defined as the time from the date of signed informed consent to first documentation of disease progression. TTP was censored at the last tumour assessment or at the date of hepatic surgery for metastatic resection or treatment discontinuation without progression due to AEs. The Kaplan-Meier method was used to calculate the TTP and OS and to estimate the hazard ratio, median values, and 95% confidence interval (CI). A subgroup analysis [including age (< 70, ≥ 70 years); baseline Karnofsky PS (60%, >60%); number of metastatic sites (1, > 1); baseline CEA (value in ng/ml); and KRAS mutation (wild type, mutation)] was performed to identify the effect of patient's baseline characteristics on response rate.

The final analyses were conducted 29 months after the last patient was included.

3. Results

3.1 Patients

Table 1. Baseline patient characteristics

Patient characteristics		**Median (range)**	**n (%)**
Age (years)		64 (40-79)	
Gender	Male		88 (73.4)
	Female		32 (26.6)
Karnofsky	60		27 (23)
	70		46 (38)
	80		39 (32)
	90		8 (7)
KRAS gene status	wild-type		64 (53)
	mutated		41 (34)
	unknown		15 (13)
Primary tumour location	Colon (except sigma)		33 (27.5)
	Sigma		42 (35)
	Rectum		45 (37.5)
Baseline metastatic disease location			
	Liver		93 (47.5)
	Only Liver		45 (37)
	Nodes		36 (18)
	Lung		31 (15.5)
	Peritoneum		30 (15)
	Bone		3 (1.5)
	Others		5 (2.5)
CEA baseline value (ng/ml)		33 (0.1-7589)	
LDH baseline value (U/l)		381 (218- 1154)	
Prior treatment	Tumour surgery (resection/derivation)		100 (83.3)
	Colon endoprosthesis		3 (2.5)
	Adjuvant or neoadjuvant chemotherapy		27 (22.5)
	Adjuvant or neoadjuvant radiotherapy		14 (11.7)

Abbreviations: CEA: Carcinoembryonic antigen; LDH: lactate dehydrogenase

The study included, between April 2005 and April 2008, 120 consecutive patients with mCRC treated at the University Central Asturias Hospital in Spain. Table 1 presents patients' baseline characteristics.

At the time of the study analysis (September 2010), 93 patients (77.5%) had died. From the remaining alive patients, 9 patients continued on study treatment and the other 18 patients had discontinued study treatment due to disease progression and were receiving second or further lines of chemotherapy.

3.2 Treatment

The median number of cycles received was 19 cycles/patient (range: 3-42), with a total of 1872 treatment cycles. Median time from informed consent signature was 0 days (range 0-10).

Seventy five patients (62.5%) required an irinotecan dose reduction and 65 patients (54.2%) required a capecitabine dose reduction. However, during the first six cycles, only 10 (8.3%) and 5 (4.2%) patients required an irinotecan or a capecitabine dose reduction, respectively. The most frequent cause of dose reduction/interruption was grade ≥2 diarrhoea for irinotecan and grade ≥2 hand-foot syndrome (HFS) for capecitabine. Other causes of study dose reduction/interruption were neutropenia, mucositis, and thrombocytopenia. The median tolerated irinotecan dose in the first six cycles was 240 mg/m^2 on day 1 every 3 weeks (100% of the foreseen dose intensity [DI]), and was 192 mg/m^2 if considering all cycles (80% of foreseen DI). The median tolerated capecitabine dose in the first six cycles was 850 mg/m^2 twice a day for 14 days every 3 weeks, and was 690 mg/m^2 when all of the cycles were considered (80% of foreseen DI).

A total of 111 patients discontinued study treatment. Reasons for discontinuation were: disease progression in 93 patients, surgical resection of metastases in 10 patients, unacceptable AEs in 7 patients, and death due to arrhythmia which was not related to the study medication in 1 patient.

In total, 81 (86.2%) out of the 93 patients with documented disease progression received subsequent chemotherapy, of which 30, 34, 12, and 5 patients received two, three, four, and five additional lines of treatment, respectively. All these patients received oxaliplatin as second-line chemotherapy, most (n=66; 81.5%) in combination with capecitabine. Third line of treatment consisted of irinotecan in combination with cetuximab or panitumumab in wild-type KRAS patients.

3.3 Efficacy

All the 120 patients were evaluated for response. The ORR was 63.3% (76 patients; 95% CI: 53.97%-71.77%). A CR was observed in 3 patients (2.5%) and a PR was observed in 73 patients (60.8%). Stable disease was achieved in 36 patients (30%) and disease progression was observed in the remaining 8 patients (6.7%) with disease control in 112 (93.3%). Higher response rates were achieved in younger patients (64% vs. 60% for aged <70 and ≥ 70 years, respectively), with better Karnofsky PS (51.8% vs 66.6% for PS $\geq$ 60% and $<$ 60%, respectively) and with wild-type KRAS status (68.8% with wild-type KRAS vs. 56% with mutant KRAS), although differences between groups were not statistically significant ($p>0.05$).

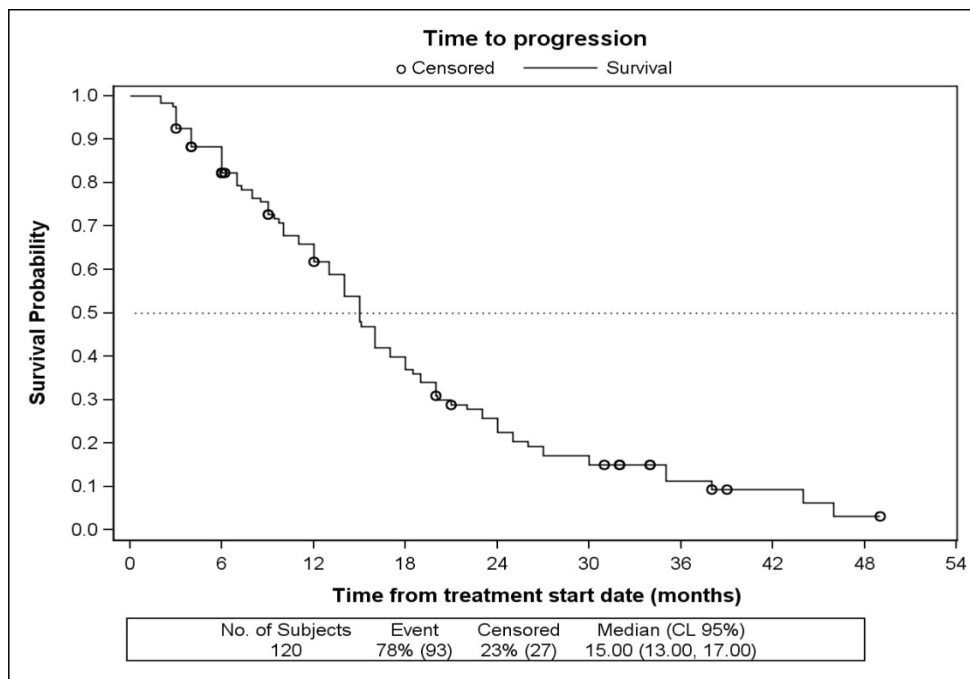

Figure 1. Kaplan-Meier estimates of time to progression

The median TTP was 15 months (range: 2-49 months; 95% CI: 13.00, 17.00 months; Figure 1)

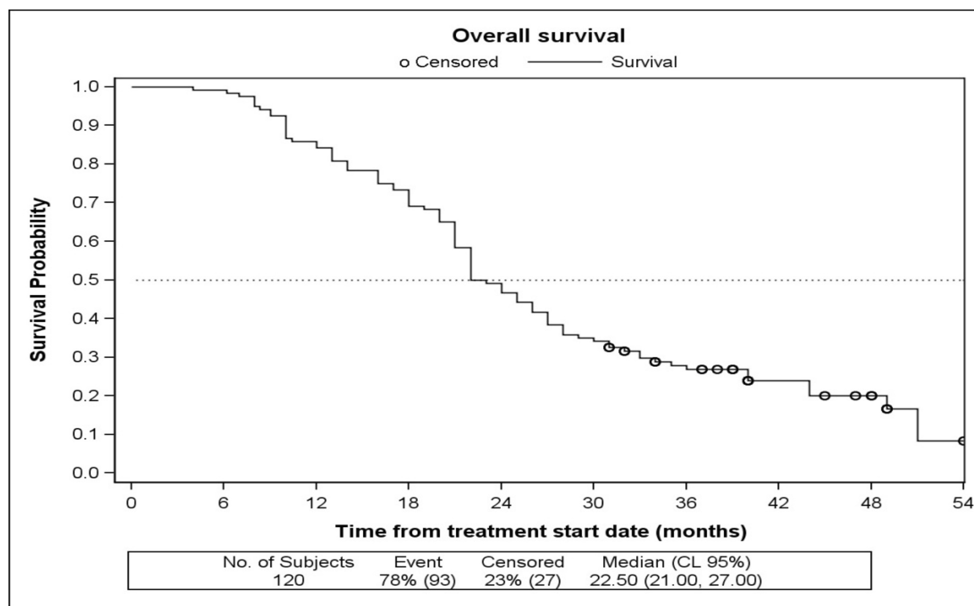

Figure 2. Kaplan-Meier estimates of overall survival

The median OS was 22.5 months (range: 4-54 months; 95% CI: 21.00, 27.00 months). The one year survival rate was 81.5%.

3.4 Liver Metastases Resection

After a median of six cycles of chemotherapy (range: 4-9 cycles), complete liver metastatic resection with curative intention was attempted in 10 (22.2%) out of the 45 patients with unresectable liver metastases at baseline.

The median length of postoperative hospitalization was 10 days (range, 8-29 days). R0 resection was feasible in all 10 patients. Survival rates at one and two year after surgery were 100% and 90%, respectively. Two patients

were disease-free at 38 and 46 months after surgery. Eight patients had recurrence after a median of 18 months (range: 6-43 months) following surgery. Wound healing postoperative complications prolonging hospitalization were reported in 2 patients. Both patients resumed study treatment.

3.5 Safety

Eighty nine patients (74.17%) were reported to have at least one treatment related AE. In total, 508 different treatment-related AEs were documented, being the majority (427 out of 508; 84.1%) with maximum CTC grade ≤2. Table 2 summarizes the incidence of treatment-related AEs.

Table 2. Maximum CAPIRI-bevacizumab related AE per patient according to NCI-CTC grade (n=120)

NCI-CTCAE AEs	Grade 1/2 n (%)	Grade 3/4 n (%)	All grades n (%)
Alopecia	42 (35.0)	35 (29.2)	77 (64.2)
Proteinuria	75 (62.5)	0 (0)	75 (62.5)
Hypertension	72 (60.0)	2 (1.7)	74 (61.7)
Hand-foot syndrome	54 (45.0)	2 (1.7)	56 (46.7)
Hemorrhagic events (bleeding/epistaxis)	51 (42.5)	4 (3.3)	55 (45.8)
Diarrhoea	35 (29.2)	20 (16.7)	55 (45.8)
Neutropenia	32 (26.7)	8 (6.7)	40 (33.3)
Febrile neutropenia	-	3 (2.5)	3 (2.5)
Vomiting	16 (13.3)	2 (1.7)	18 (15.0)
Mucositis	16 (13.3)	0 (0)	16 (13.3)
Acute cholinergic syndrome	16 (13.3)	0 (0)	16 (13.3)
Anaemia	7 (5.8)	0 (0)	7 (5.8)
Thromboembolic events	5 (4.2)	2 (1.7)	7 (5.8)
Thrombocytopenia	3 (2.5)	0 (0)	3 (2.5)
Wound-healing events	2 (1.7)	0 (0)	2 (1.7)
Hyperbilirubinemia	1 (0.8)	0 (0)	1 (0.8)
Febrile neutropenia	-	3 (2.5)	3 (2.5)
Total	427	76	508

Abbreviations: NCI-CTCAE = National Cancer institute Common Toxicity Criteria. Some patients reported more than one AE.

The most common (>20%) treatment-related AEs of any grade were alopecia (n=77, 64.2%); proteinuria (n=75; 62.5%), hypertension (n=74; 61.7%); HFS (n=56, 46.7%), diarrhoea (n=55, 45.8%), hemorrhagic events (n=55; 45.8%), and neutropenia (n=40, 33.3%). A total of 43 patients (35.8%) experienced grade 3/4 AEs, the most common being alopecia (29.2%) and diarrhoea (16.7%). Diarrhoea episodes were resolved after subsequent dose reduction, or treatment cycle delay.

HFS was generally rated as grade 1/2 (96.4%; 54 out of 56); none of the patients had grade ≥2 HFS prior to the fifth cycle. Similarly; hypertension, proteinuria, and epistaxis (n=51; 42.5%) were most of grade 1/2. Grade 3 hypertension was reported in only two (1.7%) patients. Sixty-six out of 74 (89%) patients with previous pharmacologically controlled hypertension presented additional episodes of hypertension, which were overall manageable with antihypertensive medication (angiotensin-converting-enzyme inhibitors, diuretics, or calcium-channel blockers). No arterial thrombotic events were reported in the seven patients with previous venous or arterial history (all of them used prophylaxis anticoagulation doses during the study). No relevant AEs were reported for any of the 21 patients (17.5%) with baseline ascites. There were no bevacizumab related episodes of gastrointestinal perforation or AEs leading to death. One or more cycles of bevacizumab were delayed because of toxicity in 15 patients [hypertension (n=2), thromboembolic events (n=7), hemorrhagic

events (n=4), and wound-healing complications (n=2)] and bevacizumab was reintroduced in 13 of them without further complications. Lung thromboembolism was reported in 2 patients for whom bevacizumab treatment was discontinued as per investigator decision despite the event resolution.

Six patients (5%) discontinued treatment due to treatment-related AEs, 5 of which were considered life threatening: febrile neutropenia leading to death in one patient, lung thromboembolism in two patients and, grade 4 diarrhoea with secondary renal insufficiency and dehydration in two other. The sixth AE leading to discontinuation was a grade 3 hypertension not manageable with oral antihypertensive treatment. One patient was hospitalized due to an opiate intoxication not related to study treatment but for which study treatment was firstly delayed and finally discontinued.

A total of 93 deaths were reported during the study, of which 91 were due to disease progression and two as a result of an AE which was considered treatment-related in one case. This latter was due to febrile neutropenia and occurred during the first 60 days of treatment. None died as a result of progressive disease in the first 90 days.

4. Discussion

The present observational study provides good evidence of the efficacy and good tolerance of the addition of bevacizumab to a reduced dose of CAPIRI in a 3-week schedule for the treatment of patients with mCRC in the first-line setting.

The efficacy results support the adequacy of the CAPIRI low dose regimen (irinotecan dose intensity of 80 mg/m^2 per week) in combination with bevacizumab with an ORR (63.3%), TTP (median 15 months) and OS (median 22.5 months) which are consistent with those reported in other studies with CAPIRI at low doses plus bevacizumab (Ardavanis et al., 2008; Moehler et al., 2009). Moreover, median OS observed in our study was among the range of OS rates (22.5-25.1 months) reported with FOLFIRI (irinotecan dose intensity of 90 mg/m^2 per week) regimen plus bevacizumab (Sobrero et al., 2009) or with bevacizumab plus other routine first-line chemotherapy regimens (Grothey et al., 2008; Van Cutsem et al., 2009).

Although a comparison of results from different phase studies can be only speculative, the efficacy of our schedule is in line with that obtained with oxaliplatin-based regimens combined with capecitabine with/without bevacizumab such as the TREE-2 phase II study (Hochster et al., 2008) or the NO16966 phase III study by Saltz et al. (2008) that showed lower response rates (46% and 49%, respectively) but a similar survival rate (24 and 21.3 months, respectively).

Recent studies on the integration of capecitabine-based regimens with other biologic agents (such as cetuximab) had yielded similar efficacy rates as well. A recent randomized phase II study (AIO KRK 104) in first-line treatment of mCRC found an ORR of 46% for CAPIRI plus cetuximab versus 48% for CAPOX plus cetuximab (Moosmann et al., 2011). The lack of an external control of the radiologic evaluations in our study might partly explain the good results observed in our study. In a recent single-institutional open-label phase II study of irinotecan in combination with capecitabine (XELIRI) plus bevacizumab an ORR of 67.4%, a median PFS of 12.3 months, and a median OS of 23.7 months was found (Garcia-Alfonso et al., 2010).

Different fluorouracil-based treatment regimens with irinotecan have been previously evaluated, and have overall shown a more favourable cumulative toxicity profile and convenience with low doses of CAPIRI (Cartwright et al., 2005; Kim et al., 2005; Park et al., 2004). However, the optimal dosing of CAPIRI has not been fully established. Two international studies (BICC C and EORTC 40015) using high doses of irinotecan (250 mg/m^2) plus capecitabine (2000 mg/m^2 daily for 14 days) given 3-weekly (XELIRI) resulted in an unacceptable level of toxicity (Fuchs et al., 2008; Kohne et al., 2008). Later studies with CAPIRI at lower doses (capecitabine 800-1000 mg/m^2 twice a day for 14 days and irinotecan 200-240 mg/m^2 IV on day 1 every 3 weeks) (Cartwright et al., 2005; Kim et al., 2005; Park et al., 2004), obtained a therapeutic activity similar to that observed with FOLFIRI as well as an acceptable safety profile, particularly when bevacizumab was added to the regimen (Ardavanis et al., 2008; Moehler et al., 2009). In fact, doses as low as 800 mg/m^2 twice a day for capecitabine and 200 mg/m^2 for irinotecan have been proposed as a starting point for future trials based on the regional differences observed in a review of previous studies with capecitabine-irinotecan regimens (Cartwright et al., 2010). Our regimen with little higher doses produces acceptable efficacy with manageable toxicities for most patients. Moreover, lower starting doses of irinotecan and capecitabine can be considered, since in those patients requiring dose reduction of irinotecan (62.5%) and capecitabine (54.2%) the proportion of objective responses was high.

Overall, the safety profile of bevacizumab in combination with irinotecan reported in our study is consistent with that observed in previous clinical studies. (Kozloff et al., 2009; Nalluri et al., 2008; Scappaticci et al., 2007; Scartozzi et al., 2009; Van Cutsem E. et al., 2009). The administration of irinotecan every 3 weeks did not seem to increase the toxicity. In our study a slightly higher incidence of proteinuria, hypertension, and grade 1 bleeding was observed in patients receiving bevacizumab for more than one year. However, a low incidence of grade 2/3 bevacizumab-related AEs and no bevacizumab-related gastrointestinal perforations, grade 4 AEs, or AEs leading to death were seen. Furthermore, bevacizumab did not significantly increase the occurrence of CAPIRI AEs. Moreover, a metaanalysis (Golfinopoulos et al., 2007) of 242 studies in a total of 56,677 patients substantiates the use of irinotecan based regimen plus bevacizumab as first line treatment with a significant improvement in survival when this regimen was used (it was estimated a 8 months prolongation of the absolute survival benefit) which was higher than the benefit obtained with the oxaliplatin based regimen plus bevacizumab as first line treatment (4.7 months of survival prolongation).

In our study, bevacizumab plus CAPIRI allowed potentially curative resection in 10 out of the 45 patients (22.2%) with unresectable hepatic metastases at the time of diagnosis that were deemed resectable after chemotherapy. This percentage of liver resections was similar to other studies (Okines et al., 2009; Van Cutsem et al., 2009; Yoo et al., 2006).

This prospective non-interventional study was performed in one site, which constitutes one important limitation. However, our unselected population based study included 23% patients with Karnofsky 60%, 16.6% patients >70 years and 17.5% patients with ascitis who are usually excluded in clinical trials but which is a representative sample of what is seen in common clinical practice (Hutchins et al., 1999). In addition, the possibility of investigator bias must be always considered due to the nature of the study; non-interventional, single arm, open label and non-randomised. In our study, the lack of a control group for comparison constitutes another study limitation and leaves a room for further improvement.

5. Conclusion

In summary, this study provides evidence of the clinical benefit of bevacizumab, when combined with CAPIRI at low doses, in chemonaïve patients with stage IV colorectal cancer treated in a common clinical practice setting. The use of capecitabine instead of 5-FU infusion can reduce the number of visits to day hospital, while the use of irinotecan instead of oxaliplatin as first-line treatment will possibly avoid the cumulative oxaliplatin doses, thus, reducing neurotoxicity throughout the disease course.

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Preoperative Versus Postoperative Radiation Therapy in Patients with Soft Tissue Sarcomas

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Abstract

Purpose: To evaluate treatment outcome and to determine whether or not the timing of radiotherapy (RT) was associated with any difference in disease relapse, survival, or incidence of complications in patients with soft tissue sarcomas (STS). **Methods:** The medical files of 63 patients with a primary, nonmetastatic, STS, treated with surgery and irradiation were evaluated. Data regarding tumor stage, grade, site, dosage and timing of radiotherapy, treatment complications, disease relapse, and disease-free (DFS) and overall survival (OAS) rates were analyzed. **Results:** The median follow up was 47 months (range; 5-66 months). Four-year OAS and DFS rates were 82.6% and 78.8% respectively. There were significant higher 4-year OAS ($p = 0.024$) and DFS ($p = 0.011$) rates in patients with stage I and II diseases than those in patients with stage III disease. On the other hand, there were no significant differences in 4-year OAS ($p = 0.83$, HR: 0.743, 95% CI: 0.165 to 3.345) and DFS ($p = 0.64$, HR: 0.74, 95% CI: 0.21 to 2.61) rates between preoperative and postoperative RT patients. Disease relapse for preoperative versus postoperative RT patients was not statistically different ($p = 0.41$). Wound complications were more frequent in preoperative RT patients (25%) compared to postoperative RT patients (8%) ($p = 0.0566$ chi-square). **Conclusions:** Preoperative irradiation has not a positive impact on survival or disease relapse rates, but is associated with high wound complication rate.

Keywords: soft tissue sarcomas, adjuvant radiotherapy, survival

1. Introduction

Soft tissue sarcomas represent a large group of lesions that are often subtle in presentation and have wide variation in extent of aggressive or malignant behavior (Pisters et al., 2000; Weiss et al., 2001; Sondak & Chang, 2001).

The principal management of localized soft tissue sarcomas is surgery in order to achieve a negative margin. Local excision alone of soft tissue sarcoma resulted in local recurrence rate of 50-70% (Fuller, 2001). Therefore, adjuvant radiotherapy has a proven benefit in the treatment of soft tissue sarcoma, and results in a decrease in disease relapse. Radiation may be given either before or after surgery. However, there is lack of evidences regarding the relative effectiveness of preoperative in comparison with postoperative RT (Zagars et al., 2003).

The timing of adjuvant radiation therapy and surgery is often determined by institution preference. Pre-operative radiation therapy results in tumor shrinkage and reducing the risk of seeding at the time of surgery with facilitating surgical resection. On the other hand, postoperative radiotherapy has the advantages of no delay in definitive surgery, less wound complication and no interference with pathological analysis of the resection specimen (Fuller, 2001). Despite a low overall incidence, STS is a fairly common entity in radiation oncology clinics as level-one evidence from several randomized controlled trials supports a multidisciplinary approach (Yang et al., 1998; Prendergast et al., 2010).

This study was done to evaluate pathological and clinical patterns and treatment outcome of patients with soft tissue sarcomas as well as to determine whether or not the timing of radiotherapy was associated with any difference in disease relapse, survival, or incidence of surgical wound complications.

2. Methods

2.1. Study Subjects

This retrospective study was carried out by analyzing medical records of patients with the pathological diagnosis of soft tissue sarcomas (n=63), seen at the Surgical oncology and Radiotherapy Departments, SECI, Assiut University during the period from January 2006 until January 2012. Informed consent was obtained for all patients and the treatment decision was approved by the Institutional Review Board at our center.

Eligible patients had histologically confirmed, non metastatic soft tissue sarcomas, treated by wide local excision, with removal of a normal tissue margin from within the same muscle compartment, with negative surgical margins and radiation therapy. For patients with extremity lesions, limb sparing surgery was done. For patients with retroperitoneal sarcomas, removal of all gross disease was done, while sparing adjacent viscera not invaded by tumor. Abdominal hysterectomy, bilateral salpingo-oophorectomy, and pelvic and periaortic selective lymphadenectomy was performed in 4 patients with leomyosarcoma of uterus. In one patient with sarcoma of urinary bladder, partial cystectomy was performed as the lesion at the dome of the bladder less than 5 cm in diameter.

Data regarding age, sex, site, pathological type, tumor size, histologic grade, tumor stage, dosage and timing of radiotherapy, treatment complications, disease relapse, and disease-free interval, and last follow up were analyzed.

2.2 Radiotherapy Techniques Used

In the pre-operative setting, 3-D planning was done, with patients in supine position [except in patients with back lesions, where prone position was used]. Multiple CT cuts were taken throughout target volume at 0.5 cm intervals. At each CT slice, clinical target volume (CTV) [2 cm margin on tumor mass] and critical structures were defined. Tumor dose was 50 Gy, 2Gy/fraction, over 5 weeks, prescribed at the isocenter. In the postoperative setting, 2-D planning was done with CTV with a margin of 5cm length and 2 cm transverse on the tumor bed to tumor dose of 50 Gy, 2 Gy/fraction, then, conedown to 2cm margin on surgical scar was done to total dose of 64 Gy, using 6 MV photon beam. In patients with extremity lesions, a 1cm skin strip was preserved to avoid lymphedema.

2.3 After-Therapy Monitoring

Follow-up examinations were performed routinely monthly after treatment. MRI of the affected body site to detect local relapse and CT chest to scan for lung metastases were done annually.

2.4. Statistical Methods

The study cutoff point was January, 01, 2012. Overall survival was defined as the interval from enrollment to the date of death from any cause or last follow-up. Disease-free survival was defined as the interval from enrollment of patients, to the date of disease relapse, death from any cause or last follow-up. Disease free survival and OAS rates were estimated using Graphed prism program. The Log- rank test was used to examine differences in DFS and OAS rates. Chi-square test was used to compare percentages of wound complication and disease relapse rates in both RT groups.

3. Results

3.1 Patients' Characteristics

This retrospective study included 63 patients with soft tissue sarcomas. Median age was 40 years (range; 19-69 years). Forty one patients (65%) were males and 22 (35%) were females with male to female ratio of 1.9: 1. Twenty patients (32%) presented with lesions in lower extremities, 16 patients with lesions in trunk (25%) and in pelvis (25%), and 11 cases (18%) with lesions in upper extremities. The most common pathological type was MFH (18 patients; 28.6%), followed by FS, (17 patients; 27%), SS (12 patients; 19%), and RMS (8 patients; 13%). The majority of our patients had grade II disease (32 patients; 51%), tumor >5cm (53 patients; 84%), and stage II disease (34 patients; 54%). Preoperative radiation therapy was given in 24 patients (38%) [16 patients with pelvic lesions, 6 with abdominal lesions and 2 with thigh lesions], and postoperative radiation in 39 patients (62%) (Table 1).

Table 1. Patients' characteristics of 63 patients with soft tissue sarcomas

Patients' Characteristics	NO (%)
Age	
Median	40 years
range	19-69 years
Gender	
Male	41 (65.1%)
female	22 (34.9%)
Sites	
Upper extremities (shoulder, arm, forearm, axilla)	11 (17.5%)
Lower extremities (thigh, leg)	20 (31.7%)
Pelvis	16 (25.4%)
Trunk	16 (25.4%)
Histopathology	
Malignant fibrous histocytoma (MFH)	18 (28.6%)
Fibrosarcoma/spindle cell sarcoma (FS)	17 (27%)
Synovial sarcoma (SS)	12 (19.1%)
Rhabdomyosarcoma (RMS)	8 (12.7%)
Liposarcoma (LS)	3(4.7%)
Leomyosarcoma of uterus	4(6.3%)
Sarcoma of urinary bladder	1(1.6%)
Histologic grade	
G1	9 (14.3 %)
G2	32 (50.8 %)
G3	22 (34.9 %)
Tumor size	
<5 cm	10 (15.9%)
≥5 cm	53 (84.1%)
Disease stage	
Stage I	9 (14.3%)
Stage IIa	7 (11.1%)
Stage IIb	27 (42.9%)
Stage III	20 (31.7%)
Radiotherapy given	
Preoperative RT	24 (38.1%)
Postoperative RT	39 (61.9%)
Total	63(100%)

G: grade; RT: radiotherapy

3.2 Survival Analysis

The median follow up was 47 months (range; 5-66 months). Four-year OAS and DFS rates were 82.6% and 78.8% respectively. Table 2 showed that, 4-year OAS and DFS rates did not significantly influenced by patients' age (p=0.37, HR: 1.89, 95% CI:0.47 to 7.61and p= 0.27, HR: 1.95, 95% CI:0.59 to 6.44, respectively), patients'

gender (p = 0.71, HR: 1.34, 95% CI: 0.299 to 6.024 and p = 0.79, HR: 1.187, 95% CI: 0.33 to4.23, respectively), histopathological type (p = 0.62 and p = 0.25, respectively), and tumor size (p=0.17, HR: 3.5, 95% CI: 0.579 to 21.21 and p= 0.116, HR: 3.46, 95% CI: 0.737 to 16.23, respectively). Disease stage and grade were the only adverse prognostic factors. The 4-year OAS rates were 100%, 92.5% and 61% for patients with grade I, grade II, and grade III disease, respectively (p = 0.0797), while the 4-year DFS rates were 100%, 89.7%, and 55.5% respectively (p=0.016). The 4-year OAS rates were 100%, 100%, 91% and 54% for patients with stage I disease, stage IIa disease, stage IIb and stage III disease respectively (p=0.024), while the 4-year DFS rates were 100%, 100%, 87.8%, and 49% respectively (p = 0.011). Figures (1-4) illustrate both OAS and DFS rates in patients with STS according to adverse prognostic factors (i.e. disease stage and histologic grade). In the preoperative RT patients, 4-year OAS and DFS rates were 88%, and 86.5%, respectively compared to 80% (p = 0.83, HR: 0.743, 95% CI: 0.165 to 3.345) and 76% (p= 0.64, HR: 0.74, 95% CI: 0.21 to 2.61) respectively, for postoperative RT patients. Figures (5, 6) illustrate OAS and DFS rates for patients according to timing of adjuvant radiation therapy and surgery, with preoperative RT resulted in higher survival rates than postoperative RT, but the survival differences were not statistically significant (p > 0.05).

Table 2. Univariate analysis of prognostic factors that might affect 4 year OAS and DFS among 63 patients with soft tissue sarcomas

Variable	4-year OAS	P value	4-year DFS	P value
Age		**P=0.37**		**P=0.27**
≤40 years	81.4%	HR: 1.89, 95%	76.2%	HR: 1.95, 95%
>40 years	84.4%	CI: 0.47 to 7.61	81.7%	CI: 0.59 to 6.44
Gender		**P=0.71**		**P=0.79**
Males	84.8 %	HR: 1.34, 95%	81.2 %	HR: 1.187, 95%
Females	76.8 %	CI: 0.299 to 6.024	71.8 %	CI: 0.33 to 4.23
Histopathology				
MFH	81.8 %		75.6 %	
FS	76.7 %	**P=0.62**	76.7 %	**P=0.25**
SS	100 %		100 %	
RMS	72.9		60 %	
Others	85.7 %		85.7	
Tumor size		**P=0.17**		**P=0.116**
<5cm	100%	HR: 3.50, 95%	100%	HR: 3.46, 95%
≥5cm	79.6%	CI: 0.579 to 21.210	74.5%	CI: 0.74 to 16.23
Histologic grade				
G I	100%	**P=0.0797**	100%	**0.016**
G II	92.5%		89.7%	
G III	60.8%		55.5%	
Disease stage				
Stage I	100 %		100 %	
Stage IIa	100 %	**P=0.0236**	100 %	**P=0.011**
Stage Iib	91 %		87.8 %	
Stage III	54.3%		49.3%	
Radiotherapy given		**P=0.83**		**P=0.64**
Preoperative RT	88.2%	HR: 0.743, 95%	86.5%	HR: 0.74, 95%
Postoperative RT	80.4%	CI: 0.165 to 3.345	75.8%	CI: 0.21 to 2.61

G: grade; RT: radiotherapy

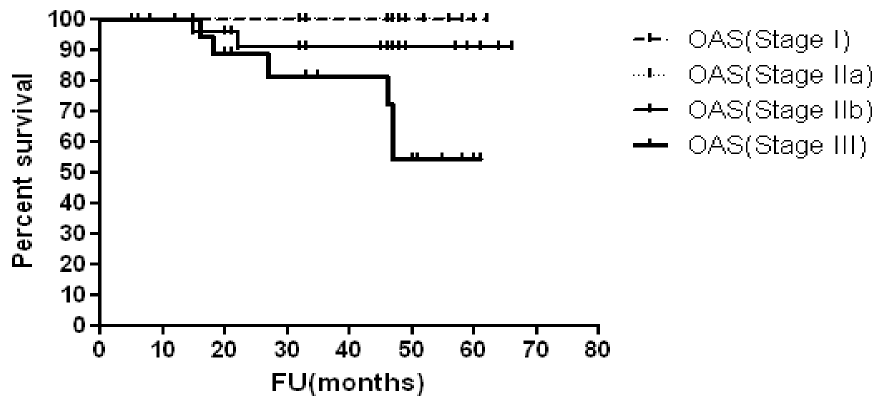

Figure 1. Overall survival curves for patients according to disease stage

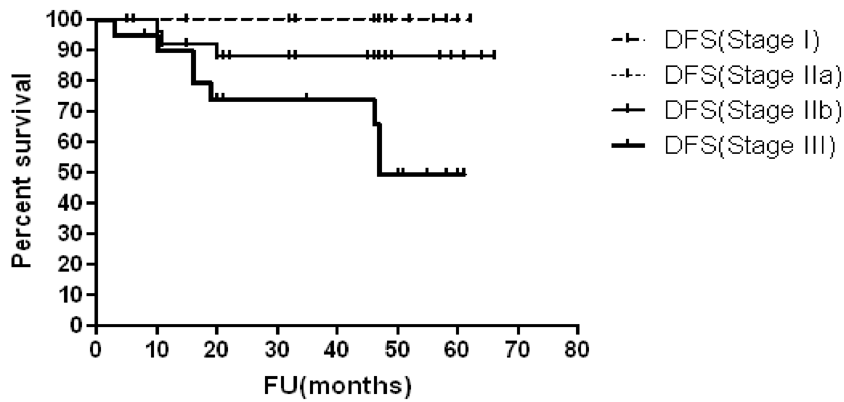

Figure 2. Disease free survival curves for patients according to disease stage

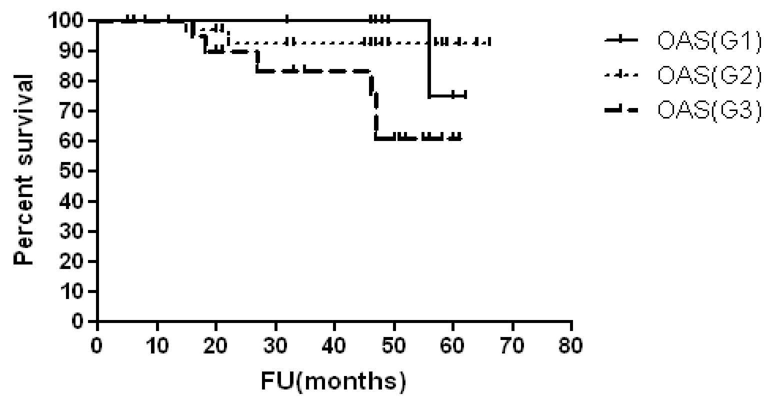

Figure 3. Overall survival curves for patients according to histologic grade

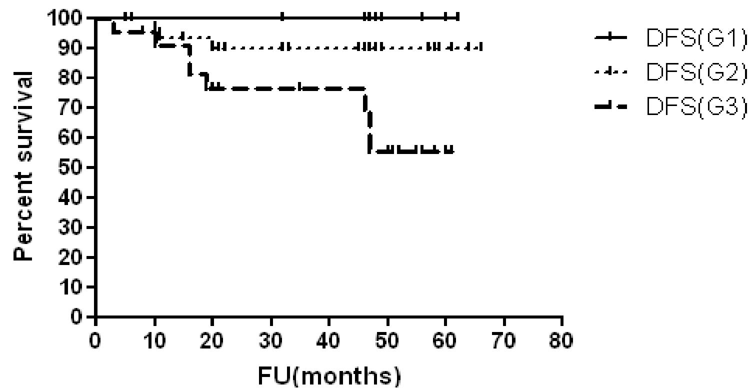

Figure 4. Disease free survival curves for patients according to histologic grade

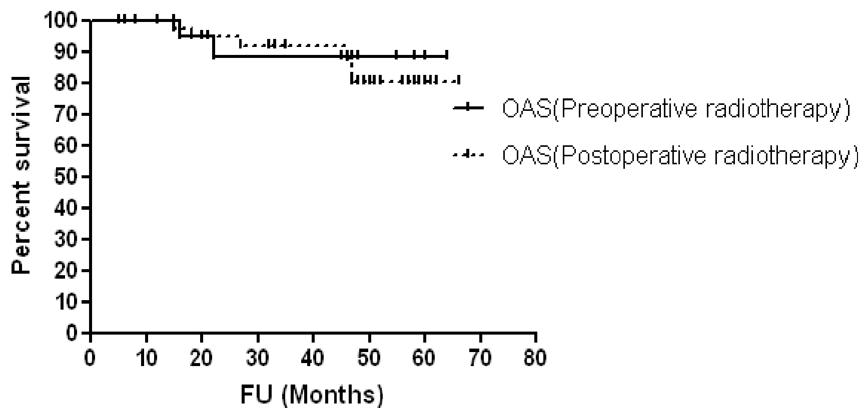

Figure 5. Overall survival curves for patients according to timing of radiotherapy

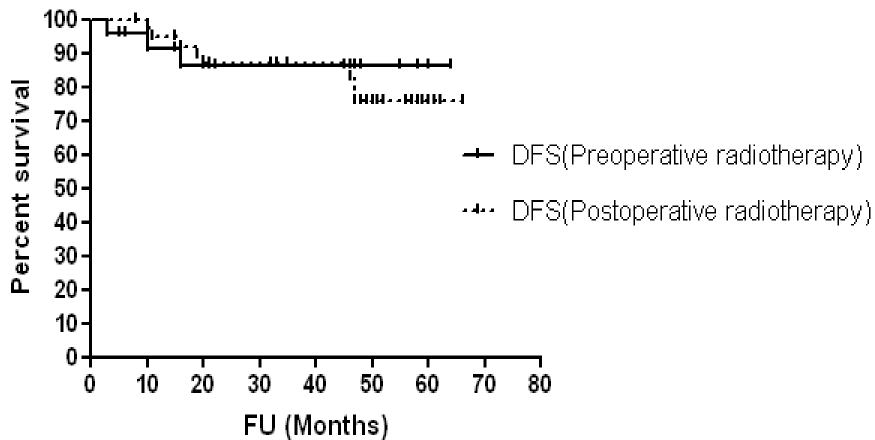

Figure 6. Disease free survival curves for patients according to timing of radiotherapy

3.3 Disease Relapses

The current study showed that disease relapse was found in 8 patients (12.7%). In the postoperative radiotherapy patients, there were 2 patients developed isolated LR, [underwent surgical excision] and 4 patients developed lung metastases [i.e. Six patients with disease relapses; 15.5%]. In the preoperative RT group, only 2 patients (8%) developed lung metastases. Patients with distant metastases (n=6), in both RT groups, were treated by salvage ifosfamide based chemotherapy. Although, disease relapse rate in the postoperative RT group was higher than that in preoperative group, there was no statistically significant difference (p=0.41) (Table 3).

Table 3. Disease relapses in soft tissue sarcoma patients who were given preoperative and postoperative radiotherapy

Variable	Preoperative radiotherapy NO (%)	Postoperative radiotherapy NO (%)	Total NO (%)	P value
Disease relapse				
Yes	2 (8.3%)	6 (15.4%)	8(12.7%)	0.41
No	22 (91.7%)	33 (84.6%)	55(87.3%)	
Total	24	39	63(100%)	

3.4 Wound Complication Rate

Complications were defined as surgical interventions for wound repair with hospital admission for wound care. Acute wound complications were more frequent in preoperative RT patients (6 patients; 25%) compared to postoperative RT patients (3 patients; 8%) (P = 0.0566, chi-square) (Table 4).

Table 4. Wound complication rate in soft tissue sarcoma patients who were given preoperative and postoperative radiotherapy

Variable	Preoperative radiotherapy NO (%)	Postoperative radiotherapy NO (%)	Total NO (%)	P value
Wound complications				
Yes	6 (25%)	3 (7.7%)	9(14.3%)	0.0566
No	18 (75%)	36 (92.3%)	54(85.7%)	
Total	24	39	63(100%)	

4. Discussion

Between 2006 and 2012, 63 patients with STS were treated at our institute, with median age of 40 years and male to female ratio of 1.9: 1. Most of the reported series showed that median age ranged between 44.5 (Kiatisevi et al., 2006), 59 years (Sternheim et al., 2011), with male predominance (van Geel et al., 2003). The most common pathological type was MFH (18 patients; 28.6%), and site was lower extremities (20 patients; 32%). In the reported series, although soft-tissue sarcomas can arise anywhere in the body, the lower extremities were the most common sites (Shmookler et al., 2001). The most common pathological type was the malignant fibrous histiocytoma (Mankin & Hornicek, 2005). The majority of our patients had grade II (32 patients; 51%), tumor >5cm (53 patients; 84%), and stage II diseases (34 patients; 54%).The reported studies showed that the most common disease grade was grade II (Van Geel et al., 2003), and tumor size was >5cm (Van Geel et al., 2003; Kiatisevi et al., 2006). A study conducted by Suit et al. (1985), showed that the most common disease stage was stage III. Regarding sequence of surgery and radiation therapy, the majority of our patients were treated by postoperative radiotherapy (62%), whereas preoperative radiotherapy was given in only 38% of patients. This is in agreement with O'Sullivan et al. (2002) where the majority of patients (55%) were treated with postoperative radiotherapy.

The present study showed that, with a median follow up of 47 months (range; 5-66 months), 4-year OAS and DFS rates were 82.6% and 78.8% respectively. This is comparable to reported survival rates as the 5-year survival rate was 74%±4% and the 10-year survival was 66%±5% (Sternheim et al., 2011).

The staging system devised by AJCC and UICC combines the most important determinants of survival in localized soft-tissue sarcomas: tumor grade, and size (Clark et al., 2005). In a univariate analysis of prognostic factors that might affect survival of patients with STS, the present study showed a trend of OAS advantage (p=0.0797) and significant DFS advantage (p = 0.016) in patients with grade I&II diseases when compared to patients with high grade disease. Regarding disease stage, the present study showed significant OAS (p=0.024) and DFS (p=0.011) advantages in patients with early disease stages when compared to patients with stage III disease. This is in agreement with reported series where high histological grade (Ramanathan et al., 1999;

Stojadinovic et al., 2002; Atalar et al., 2007) and advanced disease stage (Clark et al., 2005) were adverse prognostic factors for survival.

On the other hand, the present study showed no significant survival advantages according to patients' age and gender, histopathological type, and tumor size. Many trials have not been able to identify patients' age (Coindre et al., 1996; Cany et al., 1999), histologic type of sarcomas (Dahlberg et al., 1993; Yang et al., 1998), and tumor size (Atalar et al., 2007) as predictors of survival.

The current study showed that disease relapse was found in 8 patients (12.7%). This is confirmed by reported studies where disease relapse ranged between 9% (Prendergast et al., 2010) and 30.7% (Atalar et al., 2007).

The use of preoperative radiation has been popularized by Suit and co workers (Spiro & Suit, 1998). High rates (90 – 92%) of 10-year local control following preoperative radiation, have been reported (Brant et al., 1990). However, preparative radiation did not produce significant local control benefit when compared to postoperative radiation in the present study ($p = 0.41$) and in the reported study by investigators at the University of Minnesota (Cheng et al., 1996). Moreover, preparative radiation was associated with a significantly higher complication rate in the current study (25% versus 8%, $p = 0.0566$) and in the reported study ($p = 0.0014$) (Fuller, 2001). This is confirmed by other two studies (Cheng et al., 1996; O'Sullivan et al., 2002). The high rate of wound complication in the preoperative RT patients in the present study (25%) is comparable with complication rates (10% to 41%) found by reported studies (Eilber et al., 1995; O'Sullivan et al., 1999).

In the current study, preparative radiation did not produce significant OAS (88% versus 86%, $p=0.83$, HR: 0.743, 95% CI: 0.165 to 3.345) and DFS (80% versus 76%, $p = 0.64$, HR: 0.74, 95% CI: 0.21 to 2.61) advantages when compared to postoperative radiation. This is matched with reported studies which have revealed no significant difference in disease-free survival (Fuller, 2001), and in the overall survival (75 ± 15% versus 79 ± 11%, $p = 0.94$) (Cheng et al., 1996) between patients receiving RT preoperatively versus postoperatively.

5. Conclusion

In our retrospective study, high histological grade and advanced disease stage are found to be adverse prognostic factors for survival in patients with STS. The present study does not indicate that preoperative radiotherapy has a positive impact on survival or disease relapse rates. Moreover, it is associated with higher wound complication rates than postoperative irradiation. Prospective studies are recommended to identify subset of patients who may get benefit from preoperative irradiation.

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Is there a Diference between Brand and Generic Oxaliplatin?

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Abstract

Oxaliplatin-based chemotherapy is the preferred first-line combination chemotherapy for metastatic colorectal cancer. In the last few years the generic oxaliplatin had been introduced, some personal observation that there may be difference in the spectrum of the side effects of this compound generic and the brand preparations. The use of generic drugs has become increasingly common in clinical practice. The generic drugs are chemical medicines have simple and well-defined structures that are manufactured after the patency of the medicine is over. We collected data from two group of patient who had colorectal cancer, 1st group received the brand oxaliplatin based chemotherapy and 2nd group received the generic oxaliplatin based chemotherapy. The hematological adverse effect was not significantly different between the 2 groups but the generic oxaliplatin is associated with higher non hematological side effect than the brand oxaliplatin, both peripheral sensory neuropathy and the hand- foot syndrome. Larger prospective randomised study needed to prove these data.

Keywords: oxaliplatin, FOLFOX, colorectal cancer, chemotherapy

1. Introduction

In the late 1980s, oxaliplatin (Eloxatin) was found to have activity in advanced CRC, and it is the only platinum derivative with activity against advance colorectal cancer (CRC) (Raymond et al., 1998; Rixe et al., 1996). It is mechanism of action is that it binds and cross-links strands of DNA, forming DNA adducts—thus inhibiting DNA replication and transcription. Oxaliplatin also displays synergistic in vitro cytotoxicity with 5-FU against human colorectal cell lines (de Gramont et al., 2000). A potential mechanism for this synergism is the down regulation of thymidylate synthase by oxaliplatin, which thereby potentiates the efficacy of 5-FU (Fischel et al., 1998; Meyerhardt & Mayer, 2005). The combination of oxaliplatin plus 5-FU/leucovorin is known as the FOLFOX regimen, and it has become a standard regimen for CRC, both as adjuvant therapy and as treatment for metastatic disease (Meyerhardt & Mayer, 2005).

Oxaliplatin is a platinum derivative in which the platinum atom is complexed with1, 2 diaminocyclohexane (DACH) and with an oxalate ligand as a leaving group. It was synthesized with the goal of trying to overcome resistance to first- and second generation platinum compounds (Giacchetti et al., 2000). The use of generic drugs has become increasingly common in clinical practice. The generic drugs are chemical medicines have simple and well-defined structures that are manufactured after the patency of the medicine is over. Oxaliplatin is one of the medicine which have both the brand and the generic preparation are available in the market.

The mechanism of action of oxaliplatin is similar to that of cisplatin as well as other platinum compounds. Studies conducted to date indicate that the types and percentages of Pt-DNA adducts formed by oxaliplatin are qualitatively similar to those formed by cisplatin, but preclinical data suggest several unique attributes of the cytotoxic/antitumor activity of oxaliplatin. Oxaliplatin demonstrates a broad spectrum of in vitro cytotoxic and in vivo antitumor activity that differs from that of either cisplatin or carboplatin. Oxaliplatin is active against several cisplatin-resistant cell lines, colon carcinoma, and other solid tumours that are not responsive to cisplatin. In addition, oxaliplatin in combination with 5-FU leads to synergistic antiproliferative activity in several in vivo tumour models (Raymond, Chaney, Taamma, & Cvitkovic, 1998). Oxaliplatin has been used showed efficacy in treatment of many cancers like colon, gastric, pancreatic, and liver. In phase III studies, oxaliplatin in combination with 5-FU/LV (FOLFOX-4) has demonstrated superiority in terms of response rate and progression-free survival versus 5- FU/LV (De Gramont et al., 2000). Furthermore, the FOLFOX-4 regimen has shown superiority in RR, TTP and OS vs the Saltz regimen of irinotecan in combination with bolus 5-FU/LV. Oxaliplatin has been

preferentially developed with infusion 5-FU regimens as some safety issues surround its administration with bolus 5-FU/LV (Richard et al., 2004).

2. Methods and Patients

A retrospective study for the patients who had colorectal cancer, which had received oxaliplatin containing chemotherapy (Brand) in year 2009 and patients who had colorectal cancer who had received oxaliplatin containing chemotherapy (Generic) in year 2010, and collect the data from the source medical records and medicom data. The data included patient demography stage of the disease and the adverse effect documented for these patients 2 group patients' characteristics are compared and the spectrum of the side effects as well are compared between the 2 groups. Fifty four patients include in each group. Statistical Methods used for Data Analysis, Qualitative and quantitative values were expressed as frequency (percentage) and mean±SD. Descriptive statistics were used to summarize baseline demographic and clinical characteristics of the patients. Unpaired' T test was used to compare means of quantitative measurements between the two independent treatment groups. Chi-square test was used to examine an association of various qualitative measurements between the two treatment groups. In case of having small cell frequency Fisher's exact test was applied to examine the association for variables measured qualitatively between the two groups. P- Value smaller than 0.05 was considered as statistically significant. All statistical analyses were done using statistical packages SPSS 19.0.

3. Results

Fifty four patients were included in each group of patients with colorectal cancer received oxaliplatin containing chemotherapy protocol. Statistical analysis revealed that there was no statistically significant difference in patients various baseline demographic and clinical characteristics such as age, gender, nationality, stage of disease, Treatment, and Haematological adverse affects between the Oxaliplatin Brand and Oxaliplatin generic groups ($p > 0.05$).

The percentage of subject having WBC values less than 4 (WBC < 4x10) was higher in Oxaliplatin Brand group than Oxaliplatin Generic group (50% vs. 35.8%). However, this difference was not found to be statistically significant ($p = 0.139$) Though, it was not statistically significant, the percentage of subjects reported side effects as neuropathy, grade 2 and more by clinical assessment was higher in Oxaliplatin Generic group than Oxaliplatin Brand (11.3% vs. 7.4%; $p = 0.487$). Also, other side effect such as Hand-Foot Syndrome was found to be higher in Oxaliplatin Generic group than Oxaliplatin Brand group. But because of the small size study population it doesn't reach statistically significant values (p values 0.139 and 0.487 respectively).

4. Discussion

Oxaliplatin is an antineoplastic agent; consists of a platinum atom complexed with 1,2-diaminocyclohexane (DACH) and a labile oxalate ligand. It must undergo nonenzymatic activation before antineoplastic activity occurs.In physiologic solutions, the labile oxalate ligand presumably is displaced, forming several transient reactive complexes (e.g., monoaquo DACH platinum, diaquo DACH platinum). These complexes covalently bind to specific DNA base sequences, producing intrastrand and interstrand DNA cross-links, which are thought to inhibit DNA replication and transcription. Oxaliplatin exhibits antitumor activity against colon carcinoma in vivo and exhibits synergistic antiproliferative activity with fluorouracil (Sanofi-Synthelabo Inc., 2009; Culy, Clemett, & Wiseman, 2000). Oxaliplatin has been used showed efficacy in treatment of may cancers like colon, gastric, pancreatic, and liver. The use of the FOLFOX regimen as adjuvant treatment for colon cancer was confirmed by the Multicenter International Study of Oxaliplatin/Fluorouracil/Leucovorin in the Adjuvant Treatment of Colon Cancer (MOSAIC). This study concluded that adding oxaliplatin to a regimen of 5-FU and leucovorin improves the adjuvant treatment of colon cancer. The investigators randomly assigned 2,246 patients who had undergone curative resection for stage II or III colon cancer to receive 5-FU alone or with oxaliplatin for 6 months. The primary endpoint was disease-free survival. The result showed that the rate of disease-free survival at 3 years was 78.2% in the group given 5-FU/leucovorin plus oxaliplatin vs 72.9% in the 5-FU/leucovorin only group ($P = .002$) (Andre et al., 2004; de Gramont et al., 2003). In metastatic disease, patients treated with FOLFOX had a response rate, time to disease progression, and overall survival time that were superior to those observed with other combination chemotherapies, including IFL (irinotecan plus 5-FU) or IROX (irinotecan plus oxaliplatin). These data support the use of oxaliplatin-based chemotherapy as the preferred first-line combination chemotherapy for metastasis colorectal cancer (de Gramont et al., 2003; Meyerhardt & Mayer, 2005).

In the last few years the generic oxaliplatin had been introduced, some personal observation that there may be difference in the spectrum of the side effects of this compound generic and the brand preparations.

Table 1. Patients characteristics

Variables	Oxaliplatin Brand (N= 54)	Oxaliplatin Generic (N=53)	P-value
Age	51.46 ± 10.81	53.06 ± 11.32	0.484
Gender			
Male	34 (63.0 %)	33 (62.3 %)	0.940
Female	20 (37.0 %)	20 (37.7 %)	
Nationality			
Qatari	15 (27.8 %)	9 (17.0 %)	0.181
Non Qatari	39 (72.2 %)	44 (83.0 %)	
Stage of the Disease[1]			
II	8 (14.8 %)	9 (17.4 %)	
III	20 (37.0 %)	17 (32.6 %)	0.731
IV	26 (48.1 %)	26 (50.0 %)	
Treatment			
Adjuvant	31(57.4 %)	27 (50.9 %)	0.502
Metastatic	23 (42.6 %)	26 (49.1 %)	

The use of generic drugs has become increasingly common in clinical practice. The generic drugs are chemical medicines have simple and well-defined structures that are manufactured after the patency of the medicine is over.

Oxaliplatin is one of the medicine which have both the brand and the generic preparation are available in the market. At Al-Amal hospital we have 54 patients with colorectal cancer received chemotherapy brand oxaliplatin based in 2009, and same number in 2010 patients with colorectal cancer who received generic oxaliplatin based chemotherapy. We did retrospective study to review the records of the 2 groups of the patients and find the characteristics of the patients in both group and to compare the spectrum of the toxicities in both groups. Statistical analysis revealed that there was no statistically significant difference in patients various baseline demographic and clinical characteristics such as age, gender, nationality, stage of disease, Treatment, and Haematological adverse affects between the Oxaliplatin Brand and Oxaliplatin generic groups ($p > 0.05$) (Table 1).

Table 2. Side effects

Variables	Oxaliplatin Brand (N=54)	Oxaliplatin Generic (N=53)	P-value
Treatment			
Adjuvant	31(57.4 %)	27 (50.9 %)	0.502
Metastatic	23 (42.6 %)	26 (49.1 %)	
Hematological S E WBC < 4x109/L	27 (50.0 %)	19 (35.8 %)	0.139
Hematological S E Hb < 109/L	7 (13.0 %)	8 (15.4 %)	0.721
Hematological S E Plt < 140x109/L	23 (42.6 %)	23 (43.4 %)	0.933
Neuropathy	4 (7.4 %)	6 (11.3 %)	0.487
Other Side Effects- Hand-Foot Syndrome*	1 (1.9 %)	6 (11.3 %)	0.101†

The percentage of subject having WBC values less than 4 (WBC < 4x10) was higher in Oxaliplatin Brand group than Oxaliplatin Generic group (50% vs. 35.8%). However, this difference was not found to be statistically significant ($p = 0.139$) Though, it was not statistically significant, the percentage of subjects reported side effects as neuropathy grade 2 and more was higher in Oxaliplatin Generic group than Oxaliplatin Brand (11.3% vs. 7.4%; $p = 0.487$). The assessment of neuropathy is primarily based on neurologic clinical examination. Also, other side

effect such as Hand-Foot Syndrome was found to be higher in Oxaliplatin Generic group than Oxaliplatin Brand group. But because of the small size study population it doesn't reach statistically significant values. (P values 0.139 and 0.487 respectively) (Table 2).

These result need to be confirmed in a larger prospective randomised study.

5. Conclusion

The generic oxaliplatin is associated with higher side effect than the brand oxaliplatin; both grade 2 and more peripheral sensory neuropathy and the hand- foot syndrome. Larger prospective randomised study needed to prove these data.

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Influence of Alternative Tubulin Inhibitors on the Potency of a Epirubicin-Immunochemotherapeutic Synthesized with an Ultra Violet Light-Activated Intermediate

Influence of incorporating an internal/integral disulfide bond structure and Alternative Tubulin/Microtubule Inhibitors on the Cytotoxic Anti-Neoplastic Potency of Epirubicin-(C_3-amide)-Anti-HER2/neu Synthesized Utilizing a UV-Photoactivated Anthracycline Intermediate

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Abstract

Immunochemotherapeutics, epirubicin-(C_3-*amide*)-SS-[anti-HER2/*neu*] with an internal disulfide bond, and epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] were synthesized utilizing succinimidyl 2-[(4,4´-azipentanamido) ethyl]-1,3´-dithioproprionate or succinimidyl 4,4-azipentanoate respectively. Western blot analysis was used to determine the presence of any immunoglobulin fragmentation or IgG-IgG polymerization. Retained HER2/*neu* binding characteristics of epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] and epirubicin-(C_3-*amide*)-SS-[anti-HER2/*neu*] were validated by cell-ELISA using a mammary adenocarcinoma (SKBr-3) population that highly over-expresses trophic HER2/*neu* receptor complexes. Cytotoxic anti-neoplastic potency of epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] and epirubicin-(C_3-*amide*)-SS-[anti-HER2/*neu*] between epirubicin-equivalent concentrations of 10^{-10} M and 10^{-6} M was determined by measuring the vitality/proliferation of chemotherapeutic-resistant mammary adenocarcinoma (SKBr-3 cell type). Cytotoxic anti-neoplastic potency of benzimidazoles (albendazole, flubendazole, membendazole) and griseofulvin were assessed between 0-to-2 μg/ml and 0-to-100 μg/ml respectively while mebendazole and griseofulvin were analyzed at fixed concentrations of 0.35 μg/ml and 35 g/ml respectively in dual combination with gradient concentrations of epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] and epirubicin-(C_3-*amide*)-SS-[anti-HER2/*neu*].

Cytotoxic anti-neoplastic potency for epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] and epirubicin-(C_3-*amide*)-SS-[anti-HER2/*neu*] against chemotherapeutic-resistant mammary adenocarcinoma (SKBr-3) was nearly identical at epirubicin-equivalent concentrations of 10^{-10} M and 10^{-6} M. The benzimadazoles also possessed cytotoxic anti-neoplastic activity with flubendazole and albendazole being the most and least potent respectively. Similarly, griseofulvin had cytotoxic anti-neoplastic activity and was more potent than methylselenocysteine. Both mebendazole and griseofulvin when applied in dual combination with either epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] or epirubicin-(C_3-*amide*)-SS-[anti-HER2/*neu*] produced enhanced levels of cytotoxic anti-neoplatic potency.

Keywords: anthracycline (epirubicin), chemotherapeutic-resistant, covalent immunochemotherapeutic, cytotoxic anti-neoplastic potency, mammary adenocarcinoma, selective "targeted" delivery, benzimidazoles, griseofulvin

1. Introduction

A high percentage of aggressive and resistant forms of breast cancer over-express EGFR and/or HER2/*neu* (Cameron & Stein, 2008; Medina & Goodin, 2008; Widakowich, Dinh, de Azambuja, Awada, & Piccart-Gebhart, 2008) which is frequently associated with chemotherapeutic-resistance, elevated cancer cell survival

characteristics, and increased proliferation rates (Loew, Schmidt, Unterberg, & Halatsch, 2009; Slamon, Clark, & Wong, 1987). Resistant forms of breast cancer that over-express EGFR and HER2/*neu* are often less vulnerable to the cytotoxic potency of chemotherapeutics due to simultaneous over-expression of P-glycoprotein functioning as a non-selective trans-membrane "pump" complex for many pharmaceutical substrates (Chekhun, Zhylchuk, Lukyanova, Vorontsova, & Kudryavets, 2009; Gonzalez-Angulo, Morales-Vasquez, & Hortobagyi, 2007; Liu et al., 2010; Pasquier, Magal, Boulangé-Lecomte, Webb, & Foll, 2011; Patwardhan, Gupta, Huang, Gu, & Liu, 2010; Shen, Lee, & Gan, 2011). Monoclonal immunoglobulin or pharmaceuticals with binding-avidity for HER2/*neu* (e.g. anti-HER2/*neu*: trastuzumab, pertuzumab) (Gong et al., 2011; Phillips et al., 2008; Pandya et al., 2011; Scaltriti et al., 2011; Sliwkowski et al., 1999), EGFR (e.g. anti-EGFR: cetuximab, gefitinib) (Morgillo, Woo, Kim, Hong, & Lee, 2006; Morgillo et al., 2007; Sartore-Bianchi et al., 2009; Weickhardt, Tebbutt, & Mariadason, 2010), monoclonal immunoglobulin with dual HER2/neu and EGFR binding-avidity (e.g. anti-HER2/*neu* and anti-EGFR properties: panitumumab) (Dempke & Heinemann, 2010; Modjtahedi & Essapen, 2009; Sartore-Bianchi, 2009; Weickhardt, Tebbutt, & Mariadason, 2010), or monoclonal immunoglobulin inhibitors of other trophic receptors are all effective treatment options for forms of cancer affecting the breast, intestinal tract, lung and prostate. The obvious advantage of these therapeutic monoclonal immunoglobulins is their unique mechanism-of-action and their administration avoids many of the sequelae commonly associated with conventional chemotherapeutics. Unfortunately, most monoclonal immunoglobulin-based therapies that inhibit anti-trophic receptor function are usually only capable of promoting cytostatic properties and are almost invariably plagued by an inability to evoke cytotoxic activity sufficient to resolve most aggressive or advanced forms of neoplastic disease (Chen, Xia, & Spector, 2008; Cobleigh et al., 1999; Kute et al., 2009; Lewis Phillips et al., 2008; Lin et al., 2008; Marches & Uhr, 2004; Mitra et al., 2009; Nanda, 2007; Narayan et al., 2009; Pietras, Pegram, Finn, Maneval, & Slamon, 1998; Ritter et al., 2007; Sliwkowski et al., 1999; Vogel et al., 2002). Exceptions include scenarios where they are administered in combination with conventional chemotherapeutics or other cancer treatment modalities (García-Sáenz et al., 2008; Harris, Ward, Dobbins, Drew, & Pearson, 2011; Slamon et al., 2001). Lack of cytotoxic efficacy of the anti-trophic receptor immunoglobulins has been attributed to increases in cell-cycle G_1-arrest, increased cell transformation into states of apoptosis-resistance (Marches & Uhr, 2004) and selection for resistant sub-populations (Lewis Phillips et al., 2008; Sliwkowski et al., 1999) that is frequently complicated by reversal of tumor growth inhibition (Sliwkowski et al., 1999) and relapse trophic receptor over-expression (Pietras et al., 1998) following discontinuation of therapy.

The anthracycline class of chemotherapeutics is commonly administered for the treatment of breast cancer and many other neoplastic conditions due to their superior level of potency. One of the most common dose-limiting side effects of anthracycline administration is cardiotoxicity (doxorubicin >> epirubicin). Even with the anthracyclines a complete clinical resolution of breast cancer, (particularly resistant forms), is rarely attainable especially when utilized as a monotherapy. Combination chemotherapy regimens are almost invariably more potent in suppressing the growth and metastasis of neoplastic cell types, significantly prolonging quality-of-life, delaying the onset of disease relapse, combating chemotherapeutic resistance, extending the duration of disease remission, and facilitating complete neoplastic disease elimination. Chemotherapeutic resistance is a particularly important development that hinders successful treatment of breast cancer because approximately 20-30% of all affected cases develop metastatic brain lesions which characteristically display moderate-to-high levels refractoriness to chemotherapeutic intervention (Honig et al., 2005). Despite the advantages of combination chemotherapy regimens, they still suffer from a high frequency of toxic sequelae that can limit the extent and duration of administration (Azad, Posadas et al., 2008; Balayssac et al., 2011; Ceresa & Cavaletti, 2011; Chang et al., 2001; Iarussi, Indolfi, Galderisi, & Bossone, 2000; Raschi et al., 2010; Scully & Lipshultz, 2007; Stavridi & Palmieri, 2008; Vantelon et al., 2001; Wachters, Van Der Graaf, & Groen, 2004).

Due largely to their relatively high potency against many common neoplastic conditions, the anthracyclines have long been one of the most common chemotherapeutic classes utilized in the molecular design and synthesis of therapeutic modalities that possess properties of selective "targeted delivery with the potential of improving treatment effectiveness and reducing deposition within innocent tissues and organ systems (Coyne, Jones, Sygula, Bailey, & Pinchuk, 2011; Coyne, Ross, Bailey, & Jones, 2009; Diener, Diner, Sinha, Xie, & Vergidis, 1986; Dillman, Johnson, Ogden, & Beidler, 1989; King et al., 1999; Kratz et al., 2002; Liu, Zhao, Volk, Klohr, Kerns, & Lee, 1996; Muldoon & Neuwelt, 2003; Page, Thibeault, Noel & Dumas, 1990; Thorpe et al., 1987; Worrell et al., 1986). Covalent bonding of anthracycline chemotherapeutics to monoclonal immunoglobulin therefore collectively facilitates selective "targeted" delivery, maximizes cancer cell chemotherapeutic deposition, promotes progressive intracellular chemotherapeutic accumulation, and reduces the risk and frequency of severe sequellae. In addition, the implementation of molecular platforms that fascilitate mechanisms of selective "targeted" chemotherapeutic delivery provides opportunities for attaining additive and synergistic levels of

cytotoxic anti-neoplastic potency (Pegram, Lopez, Konecny, & Slamon, 2000; Slamon et al., 2001).

Covalent immunochemotherapeutics designed to selectively bind to external surface membrane receptors whereby the entire complex is internalized by mechanisms of receptor-mediated-endocytosis ultimately liberates the chemotherapeutic moiety through various processes within the acidic endolysosome environment (pH 5.0-5.5). A previously synthesized covalent anthracycline immunochemotherapeutic, epirubicin-[anti-HER2/*neu*] designed to contain a synthetically introduced acid-labile hydrazone (C_{13}-*imino*) bond structure did not have any detectably greater cytotoxic anti-neoplastic potency against mammary adenocarcinoma (SKBr-3) populations (Coyne, Jones, & Pharr, 2011). Similar covalent anthracycline immunochemotherapeutics with acid-labile/acid-sensitive properties reportedly afford increased or accelerated liberation (cytosol bioavailability) of only 40% of their total chemotherapeutic content within the low pH of endolysosomal environments found in many cancer cell types. In parallel with the concept of acid-sensitive anthracycline immunochemotherapeutics both epirubicin-(C_3-*amide*)-SS-[anti-HER2/*neu*] and epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] were synthesized in order to determined if the synthetic introduction of a disulfide bond structure created an enzyme or acid-sensitive covalent immunochemotherapeutic. Similar covalent maytansinoid immunochemotherapeutics with an integral disulfide bond structure have been synthesized that variably provide increases in intracellular chemotherapeutic bioavailability (Erickson et al., 2010; Kellogg et al., 2011).

In combination chemotherapy protocols the anthracyclines are frequently applied in concert with conventional tubulin/microtubule inhibitor chemotherapeutics to attain increased cytotoxic anti-neoplastic potency and improve the probability of clinically resolving breast cancer and other common neoplastic conditions (Gonzalez-Angulo, 2007; Hatzis et al., 2011; Honig et al., 2005; Hudis & Schmitz, 2004; Kim et al., 2011; Manzoni et al., 2010; Neskoviæ-Konstantinoviæ, Bosnjak, Raduloviæ, & Mitroviæ, 1996; Tang, 2009; Wong & Chiu, 2010). Conventional tubulin/microtubule inhibitor chemotherapeutics include colchicine (Levy, Spino, & Read, 1991), the vinca alkaloids (Antón et al., 2011; Dorsey et al., 2010; Honig et al., 2005; Hudis et al., 2004; Wong et al., 2010), taxanes (e.g. paclitaxel) (Chan, Miles, & Pivot, 2010; Dorsey et al., 2010; Rak Tkaczuk, 2011), podophyllotoxins (e.g. etoposide: semi-synthetic derivative) (Desbène & Giorgi-Renault, 2002; Neskoviæ-Konstantinoviæ et al., 1996), and monomethyl auristatin E (MMAE) (Naumovski & Junutula, 2010). The narrow margin-of-safety (therapeutic index) and relative lack of efficacy for colchicine restricts its wide-spread application for breast cancer treatment (Finkelstein et al., 2010; Levy et al., 1991; Terkeltaub, 2009). Administration of the vinca alkaloids like vinblastine and vincristine is often complicated by neurotoxicity (Broyl et al., 2010; Cavaletti et al., 2000; Meyer, Patte-Mensah, Taleb, & Mensah-Nyagan, 2010; Windebank, 1999), bone marrow suppression (Lowe et al., 2009; van Tellingen, Buckle, Jonker, van der Valk, & Beijnen, 2003), and emergence of therapeutic resistance patterns (Cavaletti et al., 2000; Dorsey et al., 2010; Dumontet & Sikic, 1999; He, Li, Kanwar, & Zhou, 2011; Kars, Iseri, & Gündüz, 2011; Kim et al., 2011; O'Brien et al., 2008; Svirnovski et al., 2009; VanderWeele, Zhou, & Rudin, 2004). The taxane class of anti-tubulin chemotherapeutics (e.g. paclitaxel, docetaxael) suffers from very similar disadvantages including acquired/intrinsic resistance (Antón et al., 2011; Dorsey et al., 2010; Dumontet, 1999; Kars et al., 2011) secondary to multidrug resistance protein (e.g. P-glycoprotein) over-expression (Harrison & Swanton, 2008; Overmoyer, 2008; Shen et al., 2011), hypersensitivity reactions (Feldweg, Lee, Matulonis, & Castells, 2005; Karacan, Eyüboglu, Akçay, & Ozyilkan, 2004; Lee, Gianos & Klaustermeyer, 2009; Rizzo, Spaggiari et al., 2010), hematopoietic toxicity (dose limiting feature) (Escuin et al., 2011; Manzoni, 2010; Pullarkat et al., 2009), and cumulative neurotoxicity (Cavaletti et al., 2000; Hershman et al., 2011; Windebank, 1999) that can all lead to termination of treatment protocols (Tang, 2009). Podophyllotoxins (e.g. etoposide semi-synthetic derivative) induce a moderately high frequency of dose-limiting leucopenia (Chamberlain, Tsao-Wei, & Groshen, 2006; Herzig, 1991; Neskoviæ-Konstantinoviæ et al., 1996) and gastrointestinal disturbances (nausea, vomiting, stomatitis). Monomethyl auristatin E (MMAE) is too toxic for systemic administration so it instead must be covalently bonded to a large molecular weight “platform” like immunoglobulin (e.g. anti-GPNMB/anti-CRO11/glembatumumab, anti-CD30/brentuximab).

Due to the effectiveness of conventional tubulin/microtubule inhibitors in combination with the anthracyclines, and because of their different spectrums of dose-limiting toxic sequelae, there is a distinct need to identify and evaluate alternative tubulin/microtubule inhibitors that have potent cytotoxic anti-neoplastic potency. To date, very little is known about the cytotoxic anti-neoplastic potency of the benzimidazoles (anthelmintic) or griseofulvin (anti-fungal agent) tubulin/microtubule inhibitors against aggressive and resistant breast cancer or their capacity to produce additive or synergistic levels of cytotoxic anti-neoplastic potency when applied in dual combination with covalent anthracycline immunochemotherapeutics.

2. Materials and Methods

2.1 Synthesis of Covalent Epirubicin-(C_3-amide)-[Anti-HER2/neu] Immunochemotherapeutics

2.1.1 Phase-I Synthesis Reaction for UV-Photoactivated Epirubicin-(C_3-amide) Intermediates

The C_3 monoamine group of epirubicin (3.02 x 10^{-7} mg, 1.75 x 10^{-4} mmoles) was reacted at a 5:1 molar-ratio with the amine-reactive *N*-hydroxysuccinimide ester "leaving" complex of either succinimidyl 4,4-azipentanoate (1.55 x 10^{-7} mg, 3.5 x 10^{-5} mmoles) or it's disulfide analog succinimidyl 2-[(4,4´-azipentanamido)ethyl]-1,3´ -dithioproprionate (2.67 x 10^{-7} mg, 3.5 x 10^{-5} mmoles) in the presence of triethylamine ($\geq$50 mM final concentration) utilizing dimethylsulfoxide (DMSO) as an anhydrous organic solvent system (Coyne, Jones, & Bear 2012). Separate stock solutions were used to formulate reaction mixtures of epirubicin with succinimidyl 4,4-azipentanoate, or epirubicin with succinimidyl 2-[(4,4´-azipentanamido)ethyl]-1,3´-dithioproprionate each of which was continually stirred gently over a 4-hour incubation period at 25° C in the dark and protected from light exposure. The relatively prolonged incubation period of 4 hours was utilized to maximize degradation of the ester group within any residual succinimidyl 4,4-azipentanoate or succinimidyl 2-[(4,4´-azipentanamido) ethyl]-1,3´-dithioproprionate that may not of reacted in the first 30 to 60 minutes with the C_3 monoamine group of epirubicin. The reaction can alternatively be performed with the addition of PBS (10% v/v, pH 7.4) as a substitute for trieithylamine.

2.1.2 Phase-II Synthesis Reaction for Covalent Epirubicin-(C_3-amide)-[Anti-HER2/neu] Immunochemotherapeutic Utilizing UV-Photoactivated Anthracycline Intermediates

Monoclonal immunoglobulin (anti-HER2/*neu*: 1.5 mg, 1.0 x 10^{-5} mmoles) in buffer (PBS: phosphate 0.1, NaCl 0.15 M, EDTA 10 mM, pH 7.3) was combined at a 1:3.5 molar-ratio with either the UV-photoactivated epirubicin-(C_3-*amide*) or epirubicin-(C_3-*amide*)-SS- intermediates (*Phase-1 end products*) and allowed to gently mix with constant stirring for 5 minutes at 25°C in the dark. The photoactivated group of the epirubicin-(C_3-*amide*) and epirubicin-(C_3-*amide*)-SS-intermediates were then reacted with amino acid residues within the sequence of anti-HER2/*neu* monoclonal immunoglobulin during a 15 minute exposure period to UV light at 354 nm (reagent activation range 320-370 nm) in combination with constant gentle stirring (Coyne, Jones, & Bear 2012). Residual epirubicin was removed from epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] and epirubicin-(C_3-*amide*)-SS-[anti-HER2/*neu*] applying micro-scale column chromatography pre-equilibrated in PBS (phosphate 0.1, NaCl 0.15 M, pH 7.3).

2.2 Analysis, Characteristics and Properties

2.2.1 General Analysis

Immunoglobulin concentration within preparations of epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] and epirubicin-(C_3-*amide*)-SS-[anti-HER2/*neu*] was determined by measuring absorbance at 280 nm (epirubicin-corrected absorbance at 280 nm). Epirubicin concentrations were determined by excitation at 485 nm and measurement of emission at 538 nm which was utilized in concert with a standard reference control curve generated utilizing known epirubicin concentrations (10^{-9} M to 10^{-5} M). Detection of non-conjugated "free" epirubicin concentrations contained in covalent epirubicin immunochemotherapeutic preparations was determined by chloroform extraction (Etrych, Mrkvan, Ríhová, & Ulbrich, 2007; Hempel, Schulze-Westhoff, Flege, & Laubrock, 1998; Ulbrich, Etrych, Chytil, Jelinkova, & Rihova, 2003) with the organic phase collected by pipette, evaporated to dryness under a stream of nitrogen gas, and the resulting residue dissolved in Tris buffered saline (50 mM, pH 7.4) prior to spectrophotometric analysis.

2.2.2 Non-Reducing SDS-PAGE Size Separation, Western-Blot Immunodetection, and Chemiluminescent Autoradiography

Standardized amounts and concentrations (60 μg/ml) of covalent epirubicin immunochemotherapeutics and reference control immunoglobulin fractions were combined 50/50 with an equal volume of conventional PAGE sample preparation buffer (Tris/glycerol/bromphenyl blue/sodium dodecyl sulfate) formulated without 2-mercaptoethanol. Each immunoglobulin sample (0.9 μg/well) was processed without boiling and then developed in parallel with a mixture of pre-stained reference control molecular weight markers by non-reducing SDS-PAGE (11% acrylamide, 100 V constant voltage at 3°C for 2.5 hours). Developed non-reducing SDS-PAGE acrylamide gels were then equilibrated in electrophoresis "tank" buffer devoid of methanol. Lateral transfer of SDS-PAGE separated proteins onto sheets of nitrocellulose membrane for Western (immunodetection) blots was performed at 20 volts constant voltage for 16 hours at 2°C to 3°C with the transfer manifold packed in crushed ice.

Nitrocellulose membranes with laterally transferred immunoglobulin fractions for immunodetection and chemiluminescent autoradiographic analyses were equilibrated in Tris buffered saline (TBS: Tris HCl 0.1 M, NaCl 150 mM, pH 7.5, 40 ml) at 4°C for 15 minutes followed by incubation in TBS blocking buffer solution (Tris 0.1 M, pH 7.4, 40 ml) containing bovine serum albumin (BSA 5%) applied at 2^O to 3° C for 16 hours in combination with gentle horizontal agitation. Prior to further processing nitrocellulose membranes were vigorously rinsed in Tris buffered saline (Tris 0.1 M, pH 7.4, 40 ml, n = 3 rinses).

Rinsed BSA-blocked nitrocellulose membranes developed for Western-blot immunodetection analyses were incubated with biotinylated goat anti-murine IgG (1:10,000 dilution) at 4°C for 18 hours applied in combination with gentle horizontal agitation. Nitrocellulose membranes were then vigorously rinsed in TBS (pH 7.4, 4°C, 50 ml, n = 3) followed by incubation in blocking buffer (Tris 0.1 M, pH 7.4, with BSA 5%, 40 ml). Blocking buffer was decanted from nitrocellulose membrane blots which were then vigorously rinsed in TBS (pH 7.4, 4°C, 50 ml, n = 3 rinses) before incubation with strepavidin-[horseradish peroxidase] (strepavidin-HRPO 1:100,000 dilution) at 4°C for 2 hours applied in combination with gentle horizontal agitation. Prior to chemiluminescent autoradiography nitrocellulose membranes were vigorously rinsed in Tris buffered saline (Tris 0.1 M, pH 7.4, 40 ml, n = 3 rinses). Strepavidin-HRPO treated nitrocellulose membranes were then incubated in HRPO chemiluminescent substrate (25°C; 5-to-10 mins.). Autoradiography images were acquired by exposing radiographic film (Kodak BioMax XAR radiograph film) to nitrocellulose membranes sealed in transparent ultraclear re-sealable plastic bags.

2.2.3 Mammary Carcinoma Tissue Culture Cell Culture

Human chemotherapeutic-resistant human mammary adenocarcinoma (SKBr-3) cell line was utilized as an *ex-vivo* neoplasia model and was acquired directly from American Tissue Cell Culture (ATCC) within 24 months of investigation. Mammary adenocarcinoma (SKBr-3) has been the only cell line or cell type utilized, cultivated or preserved/stored frozen in the laboratory during a period of the past 6 years and during the conduction of research investigations currently described. Characteristically, mammary adenocarcinoma (SKBr-3) uniquely over-expresses epidermal growth factor receptor 1 (EGFR, ErbB-1, HER1) and highly over-expresses epidermal growth factor receptor 2 (EGFR2, HER2/*neu*, ErbB-2, CD340, p185) at 2.2×10^5/cell and 1×10^6/cell respectively.

Populations of the mammary adenocarcinoma (SKBr-3) cell line were propagated in 150-cc^2 tissue culture flasks containing McCoy's 5a Medium Modified supplemented with fetal bovine serum (10% v/v) and penicillin-streptomycin at a temperature of 37° C under a gas atmosphere of air (95%) and carbon dioxide (5% CO_2). Tissue culture media was not supplemented with growth factors, growth hormones or other growth stimulants of any type. Investigations were all performed using mammary adenocarcinoma (SKBr-3) propagated to a $\geq$85% level of confluency.

2.2.4 Cell-ELISA IgG Binding Assay

Cell suspensions of mammary adenocarcinoma (SKBr-3) were seeded into 96-well microtiter plates in aliquots of 2×10^5 cells/well and allowed to form confluent adherent monolayers over a period of 48 hours. The growth media within individual wells was then removed manually by pipette and the adherent mammary adenocarcinoma (SKBr-3) monolayers serially rinsed (n = 3) with PBS followed by stabilization onto the plastic surfaces of microtiter plates with paraformaldehyde (4% in PBS, 15 minutes). Adherent mammary adenocarcinoma (SKBr-3) monolayers were then incubated with covalent epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] and epirubicin-(C_3-*amide*)-SS-[anti-HER2/*neu*] each formulated at gradient concentrations of 0.010, 0.025, 0.050, 0.250, and 0.500 μg/ml IgG (200 μl/well) in tissue culture growth media. Direct contact incubation of mammary adenocarcinoma (SKBr-3) with epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] and epirubicin-(C_3-*amide*)-SS -[anti-HER2/*neu*] was performed at 37° C over a period of 3 hours under a gas atmosphere of air (95%) and carbon dioxide (5% CO_2). Residual non-cell bound epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] or epirubicin-(C_3-*amide*)-SS-[anti-HER2/*neu*] was removed by serial rinsing with PBS (n=3). Development of mammary adenocarcinoma (SKBr-3) then entailed incubation with β-galactosidase-[goat anti-mouse IgG] (1:500 dilution) at 25°C for 20 hours followed by serial rinsing with PBS (n=3) to remove residual non-cell bound 2° immunoglobulin. In the final stage of cell-ELISA development mammary adenocarcinoma (SKBr-3) monolayers were incubated with the β-galactosidase substrate, nitrophenyl-β-D-galactopyranoside (100 μl/well of ONPG formulated fresh at 0.9 mg/ml in PBS pH 7.2 containing $MgCl_2$ 10 mM, and 2-mercaptoethanol 0.1 M). Absorbance within each individual well was then measured at 410 nm after incubation at 37° C for a period of 15 minutes (630 nm reference wavelength).

2.2.5 Cell Survival Assay for Measuring Covalent Epirubicin-Immunochemotherapeutic Cytotoxic Potency

Covalent epirubicin-(C_3-*amide*)- [anti-HER2/*neu*] and epirubicin-(C_3-*amide*)-SS- [anti-HER2/*neu*] immunochemotherapeutics were formulated in growth media at standardized epirubicin-equivalent concentrations of 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} M (final concentration). Individual epirubicin-equivalent concentrations of covalent epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] and epirubicin-(C_3-*amide*)-SS-[anti-HER2/*neu*] immunochemotherapeutics were then transferred in triplicate into 96-well microtiter plates containing chemotherapeutic-resistant mammary adenocarcinoma growth media 200 µl/well).

Contents within individual well of 96-well microtiter plates were removed manually by pipette at 72-hours and then the mammary adenocarcinoma (SKBr-3) monolayers were serially rinsed (n = 3) with PBS followed by incubation with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT 5 mg/ml in RPMI-1640 growth media devoid of pH indicator or bovine fetal calf serum). During a 3-to-4 hour incubation period at 37° C under a gas atmosphere of air (95%) and carbon dioxide (5% CO_2) chemotherapeutic-resistant mammary adenocarcinoma (SKBr-3) populations were allowed to biochemically convert intracellular MTT to navy-blue formazone crystals by the endogenous enzyme, mitochondrial succinate dehydrogenase. Contents of 96-well microtiter plates were then removed, and serially rinsed with PBS (n = 3) followed by dissolving the resulting blue intracellular formazone crystals with DMSO (300 µl/well). Spectrophotometric absorbance of the blue-colored supernatant was then measured at 570 nm using a computer-integrated microtiter plate reader.

3. Results

3.1 Synthetic Chemistry

During the Phase-I synthesis scheme the ester sites of succinimidyl 4,4-azipentanoate or succinimidyl 2-[(4,4′-azipentanamido)ethyl]-1,3′-dithioproprionate were reacted with epirubicin resulting in the creation of a covalent amide bond at the C_3 monoamine on the anthracycline carbohydrate moiety (daunosamine -NH_2-3’) (Figure 1). The resulting Phase-I products generated were UV-photoactivated epirubicin intermediates with or without an internal/integral disulfide bond accompanied simultaneously by the liberation of a succinimide “leaving” complex (Figure 1). In Phase-II of the synthesis scheme, the UV-photoactivated epirubicin-(C_3-*amide*) intermediates non-specifically react with chemical groups to form covalent bonds within the anti-HER2/*neu* immunoglobulin sequence (Figure 1). Epirubicin was formulated in molar excess of succinimidyl 4,4-azipentanoate or succinimidyl 2-[(4,4′-azipentanamido)ethyl]-1,3′-dithioproprionate and allowed to react over a prolonged period of time in order to maximize production of UV-photoactivated epirubicin-(C_3-*amide)* and epirubicin-(C_3-*amide)*-SS- intermediates and minimize concentrations of residual reagents.

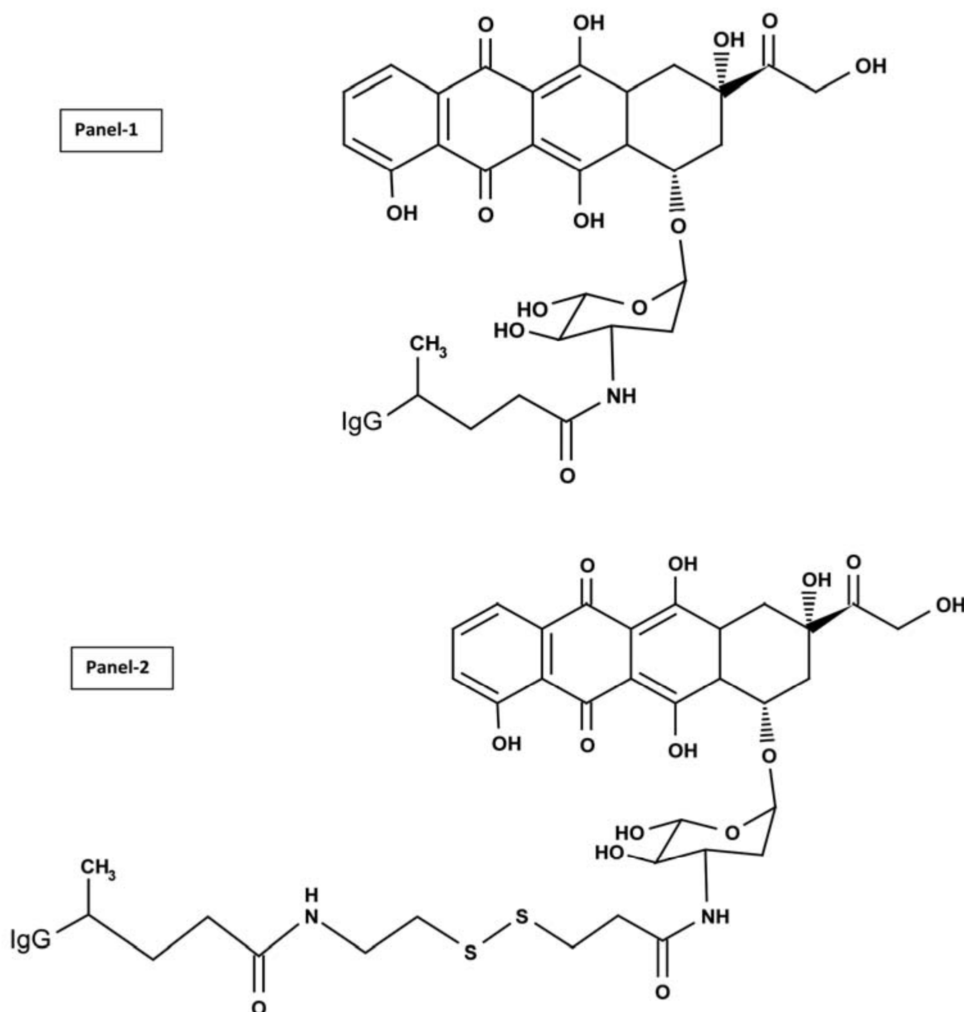

Figure 1. Molecular structure and chemical composition of two covalent epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] immunochemotherapeutics created with a 2-phase synthesis scheme using a epirubicin UV-photoactivated intermediate with and without the incorporation of an internal disulfide bond. *Top Panel-1*- covalent epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] immunochemotherapeutic synthesized utilizing succinimidyl 4,4-azipentanoate; ***Bottom Panel-2***-covalent epirubicin-(C_3-*amide*)-SS-[anti-HER2/*neu*] immunochemotherapeutic created using succinimidyl 2-[(4,4´-azipentanamido)ethyl]-1,3´-dithioproprionate which allows incorporation of an internal disulfide bond that is potentially cleavable intracellularly. *Phase-I Synthesis Scheme*: an amide bond is created at the C_3 monoamino group of the anthracycline carbohydrate moiety through the amine-reactive *N*-hydroxysuccinimide (NHS ester) group of either succinimidyl 4,4-azipentanoate or succinimidyl 2-[(4,4´-azipentanamido)ethyl]-1,3´-dithioproprionate. *Phase-II Synthesis Scheme*: epirubicin UV photoactivated intermediates create a covalent bond at amino acid residues within the amino acid sequence of anti-HER2/*neu* monoclonal immunoglobulin.

3.2 Molecular Properties

The percent of non-covalently bound anthracycline contained in covalent epirubicin immunochemotherapeutics following separation by micro-scale desalting/buffer exchange column chromatography was consistently < 4.0% of the total epirubicin content (Coyne, Jones, & Pharr 2011; Coyne et al., 2011; 2009). Residual non-covalently bound anthracycline is generally considered to not be available for further removal by serial/repeated column chromatography (Beyer et al., 2001). The anthracycline molar-incorporation-indexes for epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] and epirubicin-(C_3-*amide*)-SS- [anti-HER2/*neu*] were 40% (39.65%) and 47% (47.15%) respectively. Related preliminary analyses revealed that the epirubicin UV-photoactivated intermediates when synthesized in DMSO retained reactivity after freezing for at least 48 hours at -20°C based on an epirubicin

molar-incorporation-index of 51% when the reaction mixture was combined with bovine serum albumin at a succinimidyl 4,4-azipentanoate-to-BSA molar-ratio of 7-9:1 in concert with subsequent UV-photoactivation (354 nm, 25°C, 15 minutes). Higher epirubicin molar-incorporation-indexes are possible to achieve with modifications in methodology but the harsher synthesis conditions required for such purposes are accompanied by substantial reductions in final covalent immunochemotherapeutic yield, (Greenfield et al., 1990) and declines in antigen-immunoglobulin binding-avidity (e.g. cell-ELISA parameters). Evaluation of covalent epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] and epirubicin-(C_3-*amide*)-SS-[anti-HER2/*neu*] immunochemotherapeutics size-separated by SDS-PAGE and developed by Western blot analysis utilizing anti-murine IgG-strepavidin as a 1° immunoglobulin produced chemiluminescent autoradiography images that detected single (major) 150-kD band profiles for both the anti-HER2/*neu* reference control and each individual covalent epirubicin immunochemotherapeutic similar to results previously reported for other methodologies (Figure 2) (Coyne et al., 2011; Coyne et al., 2011b; Coyne et al., 2009; Di Stefano, Lanza, Kratz, Merina, & Fiume 2004; Sinkule, Rosen & Radosevich 1991).

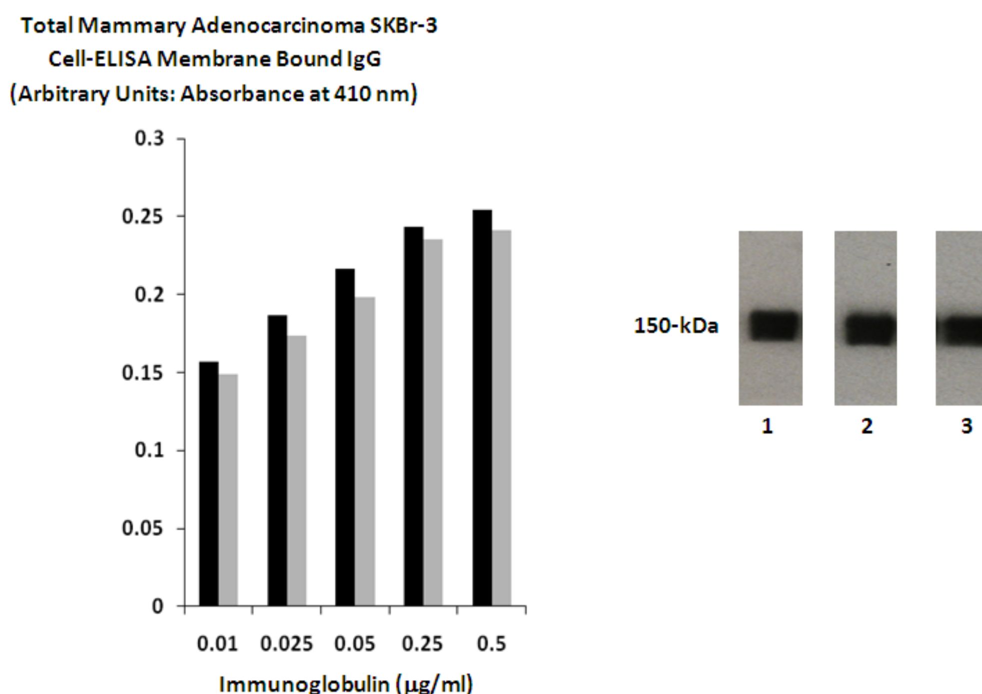

Figure 2. Biological and Physical Properties. *Left Panel 1*: Detection of total immunoglobulin bound to the exterior surface membrane of mammary adenocarcinoma in the form of covalent epirubicin immunochemotherapeutic. Legend: (■) covalent epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] immunochemotherapeutic; and (■) covalent epirubicin-(C_3-*amide*)-SS-[anti-HER2/*neu*] immunochemotherapeutic. Mammary adenocarcinoma (SKBr-3) monolayer populations were incubated with the covalent epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] immunochemotherapeutics over a 4-hour period and total immunoglobulin bound on the exterior surface membrane was measured by cell-ELISA analysis. *Right Panel 2:* Western-blot chemiluminescent autoradiography of covalent epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] and epirubicin-(C_3-*amide*)-SS-[anti-HER2/*neu*] immunochemotherapeutics. Legend: (*Lane-1*) murine anti-human HER2/*neu* immunoglobulin; *(Lane-2*) covalent epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] immunochemotherapeutic; *and (Lane-3)* covalent epirubicin-(C_3-*amide*)-SS-[anti-HER2/*neu*] immunochemotherapeutic. Immunoglobulin preparations were mass-separated by SDS-PAGE and transferred laterally onto sheets of nitrocellulose membrane to facilitate detection with biotinylated goat anti-mouse IgG. Subsequent analysis entailed incubation of nitrocellulose membranes with conjugated strepavidin-HRPO in combination with the use of an HRPO chemiluminescent substrate to facilitate the acquisition of autoradiography images.

3.3 Cell-ELISA Total Membrane IgG Binding Analyses

Epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] and epirubicin-(C_3-*amide*)-SS-[anti-HER2/*neu*] formulated at standardized immunoglobulin-equivalent concentrations of 0.010, 0.025, 0.050, 0.250, 0.500 µg/ml IgG produced cell-ELISA profiles that detected proportional increases in total immunoglobulin bound to mammary adenocarcinoma (SKBr-3) external surface membranes (Figure 2).

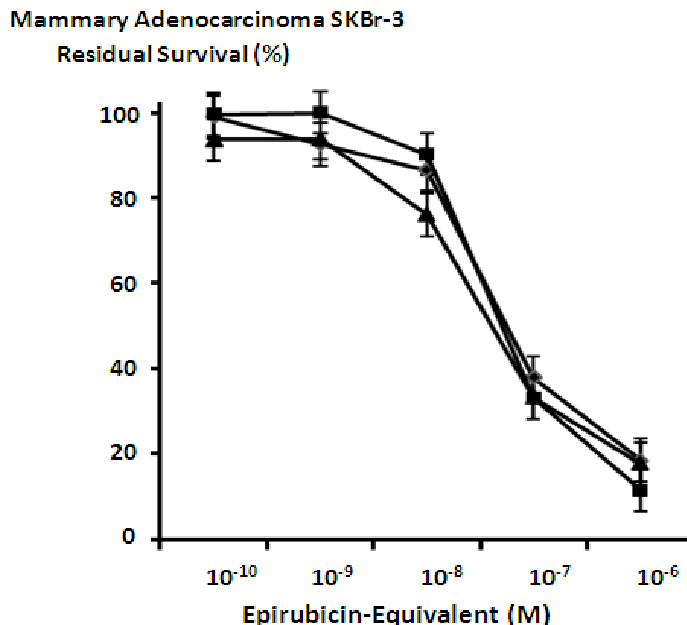

Figure 3. Influence of covalent bonding epirubicin to anti-HER2/*neu* monoclonal immunoglobulin on cytotoxic anti-neoplastic potency against chemotherapeutic-resistant mammary adenocarcinoma. Legend: (■) covalent epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] immunochemotherapeutic; (▲) covalent epirubicin-(C_3-*amide*)-SS-[anti-HER2/*neu*] immunochemotherapeutic; and (◆) epirubicin chemotherapeutic. Mammary adenocarcinoma (SKBr-3) monolayer populations were incubated with epirubicin immunochemotherapeutic for 72-hours and cytotoxicity measured as a function (%) of cell MTT vitality stain intensity (absorbance at 570 nm) relative to matched negative reference controls.

3.4. Cytotoxic Anti-Neoplastic Potency

Mean maximum cytotoxic anti-neoplastic potencies of epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] and epirubicin-(C_3-*amide*)-SS-[anti-HER2/*neu*] against chemotherapeutic-resistant mammary adenocarcinoma (SKBr-3) were 88.5% and 82.4% (11.5% & 17.6% residual survival) respectively when assessed at an epirubicin-equivalent concentration of 10^{-6} M (Figure 3). Cytotoxic anti-neoplastic potencies of epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] and epirubicin-(C_3-*amide*)-SS-[anti-HER2/*neu*] against chemotherapeutic -resistant mammary adenocarcinoma (SKBr-3) were slight greater compared to epirubicin formulated at anthracycline-equivalent concentrations but the differences in mean values were not significant (Figure 3). The covalent epirubicin-(C_3-*amide*)-SS-[anti-HER2/*neu*] immunochemotherapeutic with an internal disulfide bond produced mean cytotoxic anti-neoplastic potency levels that were not substantially different from those measured for epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] (Figure 3). Individual fractions of anti-HER2/*neu* monoclonal immunoglobulin alone did not exert any detectable anti-neoplastic activity against mammary carcinoma (SKBr-3) at the end of a 72-hour incubation period which is in accord with observations reported in previous investigations (Figure 4) (Coyne et al., 2011; Coyne et al., 2011b; Coyne et al., 2009; Dillman et al., 1989; Sapra et al., 2005; Sinkule et al., 1991; Sivam, Martin, Reisfeld, & Mueller 1995; Yang & Reisfeld 1988a).

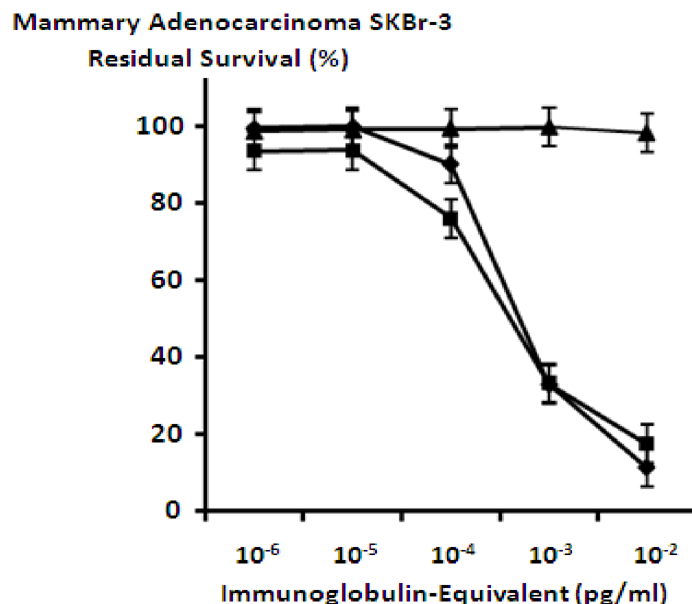

Figure 4. Relative cytotoxic anti-neoplastic potency of covalent epirubicin-immunochemotherapeutics compared to anti-HER2/*neu* monoclonal immunoglobulin against chemotherapeutic-resistant mammary adenocarcinoma. Legend: (◆) epirubicin-(C_3-*amide*)-[anti-HER2/*neu*]; (■) epirubicin-(C_3-*amide*)-SS-[anti-HER2/*neu*]; and (▲) anti-HER2/*neu* monoclonal immunoglobulin. Mammary adenocarcinoma (SKBr-3) monolayer populations were incubated with immunochemotherapeutics or monoclonal immunoglobulin over a 72-hour period and cytotoxicity measured as a function (%) of MTT cell vitality stain intensity (absorbance at 570 nm) relative to matched negative reference controls.

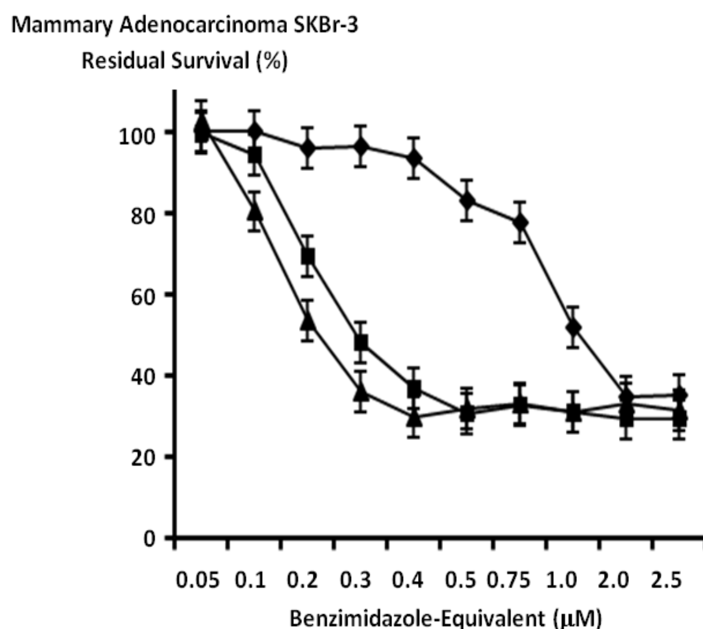

Figure 5**.** Relative cytotoxic anti-neoplastic potency of benzimidazole tubulin/microtubule inhibitors against chemotherapeutic-resistant mammary adenocarcinoma. *Legend*: (◆) albendazole; (▲) flubendazole; and (■) mebendazole. Mammary adenocarcinoma (SKBr-3) monolayer populations were incubated with individual benzimidazole tubulin/microtubule inhibitors for 72-hours and cytotoxicity measured as a function of MTT cell vitality stain intensity (absorbance at 570 nm) relative to matched negative reference controls.

The benzimadazole tubulin/microtubule inhibitors, albendazole, flubendazole and mebendazole exerted substantial cytotoxic anti-neoplastic potency against chemotherapeutic-resistant mammary adenocarcinoma (SKBr-3) at final concentrations formulated between 0.05 μM to 2.5 μM (Figure 5). Mean cytotoxic anti-neoplastic potency profiles for both flubendazole and mebendazole revealed progressive increases from near 0% and 0% at 0.05 μM to 70.2% and 63.1% (29.8% and 36.9% residual survival) at the benzimidazole-equivalent concentration of 0.4 μM (Figure 5). Mean cytotoxic anti-neoplastic potency profiles for albendazole revealed a progressive increase in cytotoxic anti-neoplastic potency from 6.2% (93.8% residual survival) at a benzimidazole-equivalent concentration of 0.4 μM, to a near maximum of 65.4% (34.6% residual survival) at 2.0 mM (Figure 5). Mean maximum cytotoxic anti-neoplastic potencies for albendazole, flubendazole and mebendazole were 64.8%, 68.7% and 70.9% (35.2%, 31.3.% and 29.1% residual survival) at a benzimidazole-equivalent concentration of 2.5 μM (Figure 5).

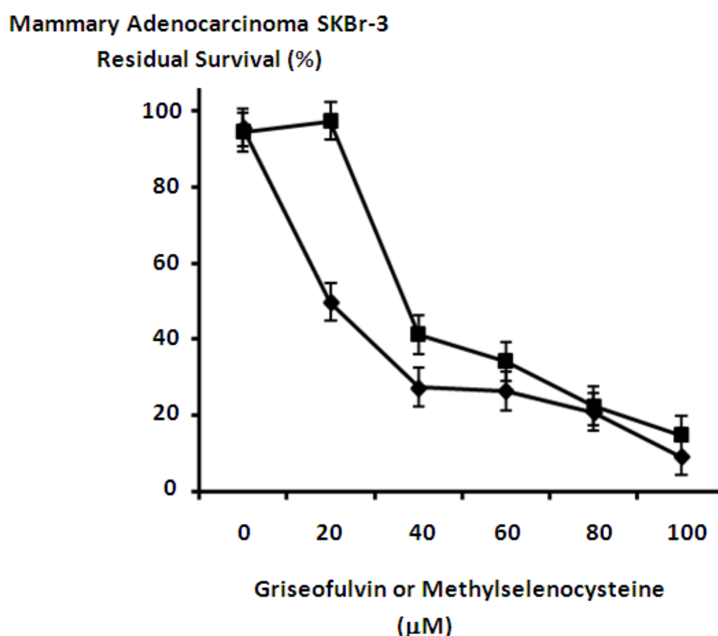

Figure 6. Relative cytotoxic anti-neoplastic potency of griseofulvin tubulin/microtubule inhibitor and methylselencysteine against chemotherapeutic-resistant mammary adenocarcinoma. *Legend*: (◆) griseofulvin; and (■) methyselenocysteine. Mammary adenocarcinoma (SKBr-3) monolayer populations were incubated with griseofulvin or methylselenocysteine for 72-hours and cytotoxicity measured as a function of MTT cell vitality stain intensity (absorbance at 570 nm) relative to matched negative reference controls.

The anti-fungal tubulin/microtubule inhibitor griseofulvin, in addition to methylselenocysteine exerted effective levels of cytotoxic anti-neoplastic potency against chemotherapeutic-resistant mammary adenocarcinoma (SKBr-3) when formulated at final concentrations between 10 μM to 100 μM (Figure 6). Increases in griseofulvin concentration to a level of 40 μM resulted in a progressive elevation in cytotoxic anti-neoplastic potency to 72.6% (27.4% residual survival) with a peak maximum level of 90.8% (9.2% residual survival) detected at 100 μM (Figure 6). Methylselenocysteine created progressive and rapid elevations in mean cytotoxic anti-neoplastic potency from 2.6% to 58.7% (97.4% to 41.3% residual survival) between the concentration range of 20 μM to 40 μM with peak levels of 85.2% (14.8% residual survival) detected at a final concentration of 100 μM (Figure 6).

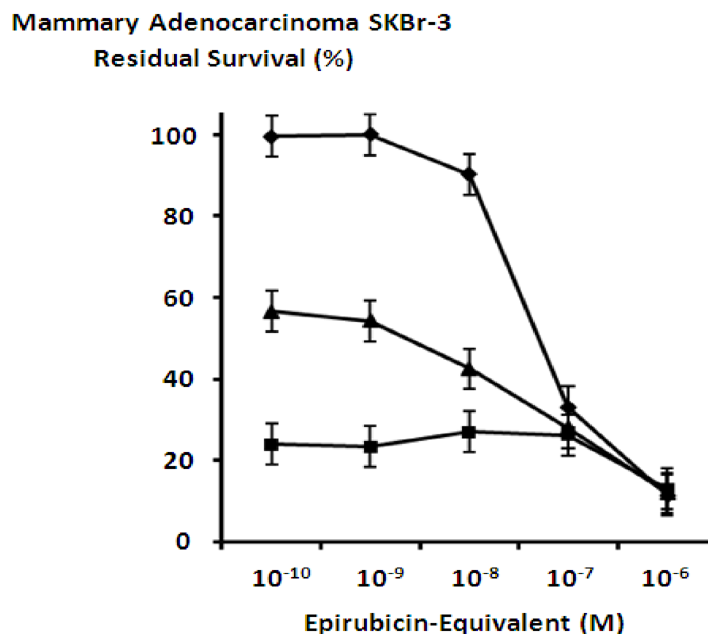

Figure 7. Collective cytotoxic anti-neoplastic potency of epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] synthesized with an epirubicin UV-photoactivated intermediate when applied in combination with mebendazole or griseofulvin tubulin/microtubule inhibitors. Legend: (◆) epirubicin-(C_3-*amide*)-[anti-HER2/*neu*]; (▲) epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] in combination with griseofulvin (15 μM fixed concentration); and (■) epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] in combination with mebendazole (0.35 μM fixed concentration). Mammary adenocarcinoma (SKBr-3) monolayer populations were incubated with the covalent epirubicin-immunochemotherapeutic in combination with the tubulin/microtubule inhibitors for 72-hours and cytotoxicity measured as a function of MTT cell vitality stain intensity (absorbance at 570 nm) relative to matched negative reference controls.

Mebendazole and griseofulvin applied in combination with either epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] or epirubicin-(C_3-*amide*)-SS-[anti-HER2/*neu*] resulted in marked increase in cytotoxic anti-neoplastic potency against chemotherapeutic-resistant mammary adenocarcinoma (SKBr-3) (Figures 7, 8 & 9). Mebendazole at a fixed final concentration of 0.35 μM applied in combination with epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] produced mean cytotoxic anti-neoplastic potency levels of 76.1% to 73% (23.9% to 27.0% residual survival), while mebendazole in combination with epirubicin-(C_3-*amide*)-SS-[anti-HER2/*neu*] produced mean cytotoxic anti-neoplastic potency levels of 68.7% and 65.0% (31.3% to 35.0% residual survival) between the epirubicin-equivalent concentration range of 10^{-10} M to 10^{-7} M respectively (Figures 7, 8 & 9). Mean peak cytotoxic anti-neoplastic potency for mebendazole in combination with epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] and epirubicin-(C_3-*amide*)-SS-[anti-HER2/*neu*] was 87.1% and 81.9% (12.9% and 18.1% residual survival) at the epirubicin-equivalent concentration of 10^{-6} M (Figures 7, 8 & 9).

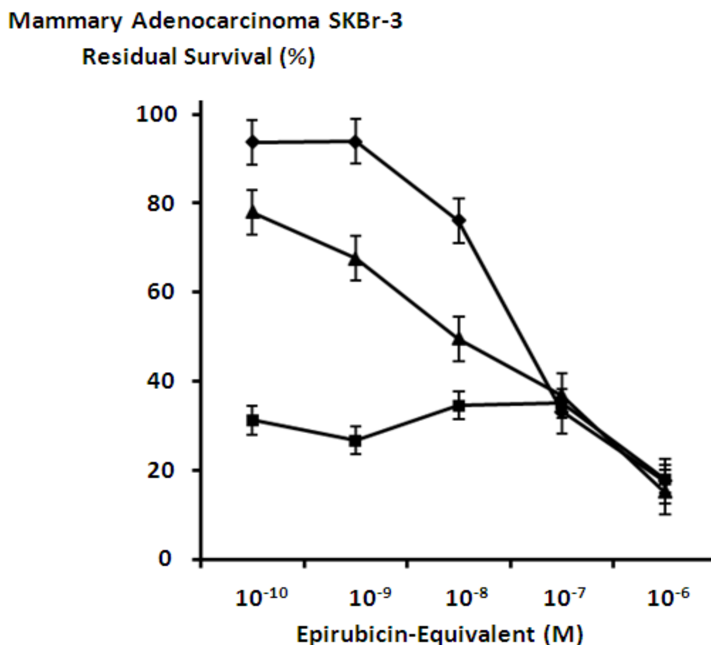

Figure 8. Collective cytotoxic anti-neoplastic potency of epirubicin-(C_3-*amide*)-SS-[anti-HER2/*neu*] synthesized with an epirubicin UV-photoactivated intermediate when applied in combination with mebendazole or griseofulvin tubulin/microtubule inhibitors. Legend: (◆) epirubicin-(C*3-amide*)-SS-[anti-HER2/*neu*]; (▲) epirubicin-(C_3-*amide*)-SS-[anti-HER2/*neu*] in combination with griseofulvin (15 µM fixed concentration); and (■) epirubicin-(C_3-*amide*)-SS-[anti-HER2/*neu*] in combination with mebendazole (0.35 µM fixed concentration). Mammary adenocarcinoma (SKBr-3) monolayer populations were incubated with the covalent epirubicin-immunochemotherapeutic in combination with the tubulin/microtubule inhibitors for 72-hours and cytotoxicity measured as a function of MTT cell vitality stain intensity (absorbance at 570 nm) relative to matched negative reference controls.

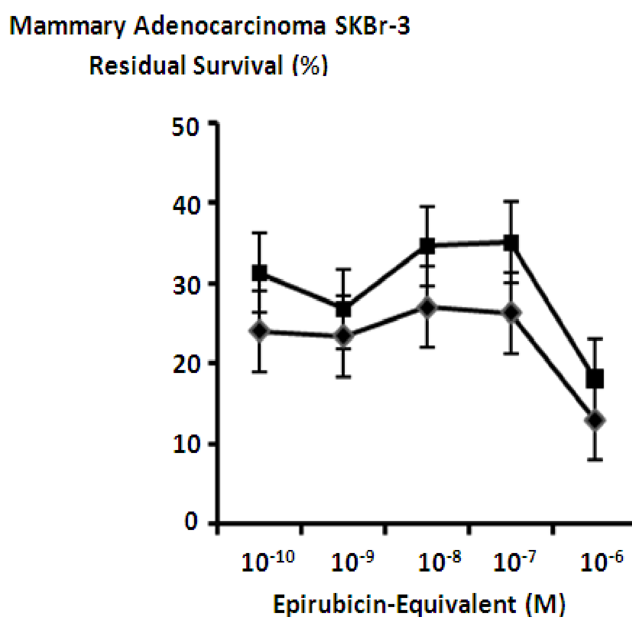

Figure 9. Collective cytotoxic anti-neoplastic potency of epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] or epirubicin-(C_3-*amide*)-SS-[anti-HER2/*neu*] synthesized with an internal disulfide bond when each covalent

anthracycline immunochemotherapeutic was applied in combination with mebendazole. Legend: (◆) epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] in combination with mebendazole (0.35 µM fixed concentration; and (■) epirubicin-(C_3-*amide*)-SS-[anti-HER2/*neu*] in combination with mebendazole (0.35 µM fixed concentration). Mammary adenocarcinoma (SKBr-3) monolayer populations were incubated with covalent epirubicin-immunochemotherapeutics in combination with benzimidazole for 72-hours and cytotoxicity measured as a function of MTT cell vitality stain intensity (absorbance at 570 nm) relative to matched negative reference controls.

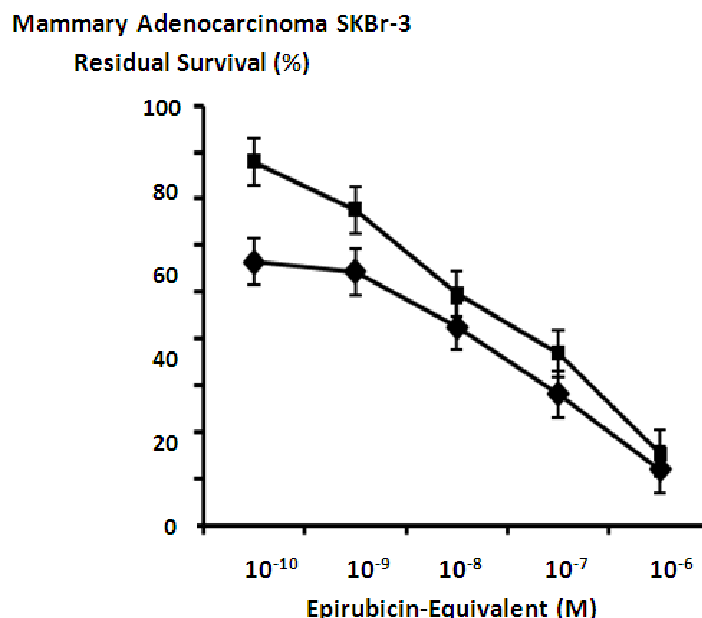

Figure 10. Collective cytotoxic anti-neoplastic potency of epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] or epirubicin-(C_3-*amide*)-SS-[anti-HER2/*neu*] synthesized with an internal disulfide bond when each covalent anthracycline immunochemotherapeutic was applied in combination with griseofulvin. Legend: (◆) epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] in combination with griseofulvin (15 µM fixed concentration; and (■) epirubicin-(C_3-*amide*)-SS-[anti-HER2/*neu*] in combination with griseofulvin (15 µM fixed concentration). Mammary adenocarcinoma (SKBr-3) monolayer populations were incubated with covalent epirubicin-immunochemotherapeutics in combination with benzimidazole for 72-hours and cytotoxicity measured as a function of MTT cell vitality stain intensity (absorbance at 570 nm) relative to matched negative reference controls.

Griseofulvin at a fixed final concentration of 35 µM applied in combination with epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] produced moderately progressive increases in mean cytotoxic anti-neoplastic potency from 45.7% to 88.0% (54.3% to 12.0% residual survival) between the epirubicin-equivalent concentration range of 10^{-9} M to 10^{-6} M respectively (Figure 7). Similarly, griseofulvin at a final fixed concentration of 35 µM applied in combination with epirubicin-(C_3-*amide*)-SS-[anti-HER2/*neu*] produced a moderately progressive increase in mean cytotoxic anti-neoplastic potency from between 22.0% to 84.7% (78.0% to 15.3% residual survival) within the final epirubicin-equivalent concentration range of 10^{-10} M to 10^{-6} M respectively (Figure 8). Mean peak cytotoxic anti-neoplastic potency levels for griseofulvin in combination with epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] and epirubicin-(C_3-*amide*)-SS-[anti-HER2/*neu*] were 88.0% and 84.7% (12.0% and 15.3% residual survival) at the final epirubicin-equivalent concentration of 10^{-6} M (Figures 7 & 8). Both mebendazole and griseofulvin in dual combination with epirubicin-(C_3-*amide*) -[anti-HER2/*neu*] evoked slightly greater cytotoxic anti-neoplastic potency than levels detected when they were applied in concert with epirubicin-(C_3-*amide*)-SS-[anti- HER2/*neu*] but this consistent trend was not considered profound (Figures 9 & 10).

4. Discussion

4.1 Immunochemotherapeutic

A number of methods have been described for synthesizing covalent anthracycline-immunochemotherapeutics, but most techniques have a lengthy duration (e.g. days), often afford low total yields, and employ dedicated organic chemistry reactions for the production of only a single immunochemotherapeutic. In this context, the heterobifunctional covalent bond forming reactants, succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) (Coyne et al., 2009; Frost, Jensen & Lindegren 2010; Karacay et al., 1997; Lau, Bérubé, & Ford 1995), N-*ε*-maleimidocaproic acid hydrazide (EMCH) (Coyne et al., 2011; Furgeson, Dreher, & Chilkoti 2006; Kruger et al., 1997), or *N*-[*p*-maleimidophenyl]-isocyanate (PMPI) (Annunziato, Patel, Ranade & Palumbo 1993; Coyne et al., 2011; Liu, de Wijn, & van Blitterswijk 1998; Wang, et al., 2009) require pre-thiolation of peptide/protein based molecular platforms (e.g. IgG, Fab', receptor ligands). Alternatively, several distinct advantages can be realized when covalent immunochemotherapeutics are synthesized utilizing succinimidyl 4,4-azipentanoate or succinimidyl 2-[(4,4′-azipentanamido)ethyl]-1,3′-dithioproprionate which most notably include the application of relatively mild reaction conditions (e.g. lower risk of inter-IgG and intra-IgG polymerization or fragmentation) (Di Stegano et al., 2004), lack of pre-thiolation requirements, implementation of fewer individual organic chemistry reactions, option of employing non-anthracycline chemotherapeutic moieties, flexibile convenience of temporary UV-photoactivated chemotherapeutic intermediate storage, higher retained biological activity, and greater end-product yield (Coyne, Jones, & Bear 2012).

4.2 Cell-ELISA IgG Membrane Binding Analysis

Profiles for total membrane-bound IgG on the exterior surface of mammary adenocarcinoma (SKBr-3) populations incubated with epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] and epirubicin-(C_3-*amide*)-SS-[anti-HER2/*neu*] increased proportionately with gradient elevations in total immunoglobulin content (Figure 2). Total cell membrane-bound epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] and epirubicin-(C_3-*amide*)-SS-[anti-HER2/*neu*] was higher than previously observed for [*i*] epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] synthesized with succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) in combination with *N*-succinimidyl -S-acetylthioacetate (SATA) (Coyne et al., 2009), [*ii*] epirubicin-(C_{13}-*keto*)-[anti-HER2/*neu*] synthesized with N-*ε*-maleimidocaproic acid hydrazide (EMCH) in combination with 2-iminothiolane (2-IT) (Coyne et al., 2011b), or [*iii*] gemcitabine-(C_5-*carbamate*)-[anti-HER2/*neu*] synthesized with *N*-[*p*-maleimidophenyl]-isocyanate (PMPI) in combination with 2-iminothiolane (2-IT) (Coyne et al., 2011). Presumably the difference in part reflects a higher degree of retained epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] and epirubicin-(C_3-*amide*)-SS-[anti-HER2/*neu*] binding-avidity for exterior surface membrane-associated HER2/*neu* because succinimidyl 4,4-azipentanoate and succinimidyl 2-[(4,4′-azipentanamido)ethyl]-1,3′-dithioproprionate do not require immunoglobulin pre-thiolation prior to the Phase-II synthesis reaction.

In addition to protein (immunoglobulin) pre-thiolation that can under certain conditions result in intra-molecular and inter-molecular disulfide bond formation, the binding-avidity of epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] and epirubicin-(C_3-*amide*)-SS-[anti-HER2/*neu*] for membrane HER2/*neu* was also likely influenced by the covalent bonding of the anthracycline moiety to different amino acid residues within the Fab' antigen binding region of anti-HER2/*neu* immunoglobulin. Seemingly modest alterations in synthetic chemistry reactions and elevations in chemotherapeutic molar-incorporation-index can profoundly influence immunoglobulin binding properties (Yang & Reisfeld 1988a). The relatively mild conditions employed during organic chemistry reaction schemes and the relatively low molar-incorporation-index of 40.0% collectively contribute to the high biological integrity of epirubicin (C_3-*amide*)-[anti-HER2/*neu*] and epirubicin (C_3-*amide*)-SS-[anti-HER2/*neu*] based on the collective interpretation of results from SDS-PAGE chemiluminescent autoradiography, cell-ELISA analyses and cytotoxic anti-neoplastic potency against chemotherapeutic-resistant mammary adenocarcinoma (SKBr-3).

4.3 Cytotoxic Potency of Covalent Epirubicin Immunochemotherapeutics

Creation of a synthetic covalent bond between epirubicin and anti-HER2/*neu* monoclonal immunoglobulin for the production of epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] or epirubicin-(C_3-*amide*)-SS-[anti-HER2/*neu*] did not decrease the cytotoxic potency of the anthracycline against chemotherapeutic-resistant mammary adenocarcinoma (SKBr-3) when assessed between the epirubicin-equivalent concentration range of 10^{-10} M to 10^{-6} M (Figures 3 & 4). Similar properties have been recognized previously for epirubicin-(C_{13}-*imino*) -[anti-HER2/*neu*] (Coyne et al., 2011), epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] (Coyne et al., 2009), epirubicin-(C_3-*amide*)-[anti-EGFR] (Coyne et al., 2009) and analogous covalent immunochemotherapeutics designed to selectively "target" anthracycline delivery (Dillman et al., 1989; Herbert, Norris, & Sauk 2003; Johnson, Briggs, Gutowski, & Barton 1995; King et al., 1999; Sivam et al., 1995; Stan, Radu, Casares, Bona, &

Brumeanu 1999; Yang et al., 1988a).

Synthetic incorporation of an internal (integral) disulfide bond into the molecular structure of covalent immunochemotherapeutics has potential merit as a strategy for enhancing the intracellular bioavailability of chemotherapeutic moieties following internalization by mechanisms of receptor-mediated endocytosis. The presumption is largely based on the concept that glutathione (GSH) tri-peptide is found intracellularly at levels that are 100x to 1000x (≅ 2-to-10 mM) higher than concentrations found within the extracellular fluid or plasma compartments (≅ 2-to-20 μM). Given this difference, disulfide bond structures have been synthetically introduced into maytansinoid-immunochemotherapeutics with the intent of improving their intracellular bioavailability following internalization by "targeted" neoplastic cell populations (Erickson et al., 2010; Kellogg et al., 2011). Due to these considerations epirubicin-(C_3-*amide*)-SS-[anti-HER2/*neu*] was synthesized with an internal (integral) disulfide bond structure in order to determine if such a chemical modification influences cytotoxic anti-neoplastic potency against mammary adenocarcinoma (SKBr-3) presumably by promoting elevations in the intracellular bioavailability of the anthracycline moiety (Figure 1). In contrast to the cytotoxic anti-neoplastic potency of covalent maytansinoid-immunochemotherapeutics against various cancer cell types like human intestinal carcinoma (xenografts) (Erickson et al., 2010; Kellogg et al., 2011), the cytotoxic anti-neoplastic potency of epirubicin-(C_3-*amide*)-SS-[anti-HER2/*neu*] was not significantly greater compared to epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] (Figure 3). Similar to results detected in comparisons between epirubicin-(C_3-*amide*)-SS-[anti-HER2/*neu*] and epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] several other covalent immunochemotherapeutics with internal disulfide bonds incorporated into their molecular structure have also been found to not possess increased levels of cytotoxic anti-neoplastic potency (Erickson et al., 2006; Lewis Phillips et al., 2008; Sun et al., 2011). Certain maytansinoid-SS-[anti-HER2/*neu*] immunochemotherapeutics in this regard that contain a synthetically introduced disulfide bond do not exert higher planes of cytotoxic anti-neoplastic potency against HER2/*neu* positive breast cancer (Lewis Phillips et al., 2008). Potency of epirubicin-(C_3-*amide*)-SS-[anti-HER2/*neu*] against mammary adenocarcinoma (SKBr-3) might have been improved if a modified analog of succinimidyl 2-[(4,4′-azipentanamido)ethyl]-1,3′-dithioproprionate had been utilized to incorporate an internal disulfide bond structure at a physically different location or in a different molecular configuration (Kellogg et al., 2011). A supportive analogy is the observation that mytansinoid-immunochemotherapeutics that contain two methyl groups in close proximity to chemotherapeutic moieties and are devoid of methyl groups on the "linker side" exert only intermediate levels of plasma stability, but superior levels of cytotoxic anti-neoplastic potency against xenografts of human intestinal carcinoma (Kellogg et al., 2011). Synthetic introduction of disulfide bond structures into covalent mytansinoid-immunochemotherapeutics can therefore increase *in-vivo* susceptibility to premature enzymatic degradation but if they are located in a sterically hindered position they are less susceptible to enzyme-mediated liberation within the intravascular compartment (Kellogg et al., 2011). Unfortunately it was not possible to evaluate the influence of disulfide bond position on the cytotoxic potency of epirubicin-(C_3-*amide*) -SS-[anti-HER2/*neu*] against mammary carcinoma due to a lack of available reagents.

Other biochemical and cell biology associated variables may also account for the lack of improved cytotoxic anti-neoplastic potency of epirubicin-(C_3-*amide*)-SS-[anti-HER2/*neu*] against chemotherapeutic-resistant human mammary adenocarcinoma (SKBr-3) compared to epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] (Figure 3). Covalent epirubicin-(C_{13}-*imino*)-[anti-HER2/*neu*] immunochemotherapeutic synthesized with an internal bond structure that reportedly has acid-labile properties does not exert significantly higher levels of cytotoxic anti-neoplastic potency against mammary adenocarcinoma (SKBr-3) than a non-acid-labile epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] immunochemotherapeutic (Coyne et al., 2011b). The fact that epirubicin-(C_3-*amide*)-SS-[anti-HER2/*neu*] also did not exert an enhanced level of cytotoxic anti-neoplastic activity against chemotherapeutic -resistant mammary adenocarcinoma (SKBr-3) may therefore reflect a cell biology related variable that explains a lack of enhanced efficacy. Interestingly, minimal or no correlation frequently exists between the *in-vitro* and *in-vivo* potency of covalent immunochemotherapeutics with synthetically introduced disulfide bond structures (Kellogg et al., 2011) which is in marked contrast to covalent immunochemotherapeutics devoid of this same internal chemical structure. Differences in cytotoxic anti-neoplastic potency to this degree *in-vivo* have been attributed to the influence of hepatic metabolization and variations in the creation of lipophilic and hydrophilic metabolites that determine the extent of distribution within fluid compartments and penetration across intact cancer cell membranes (Erickson et al., 2010) .

The cytotoxic anti-neoplastic potency of the prototypic epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] and epirubicin (C_3-*amide*)-SS-[anti-HER2/*neu*] immunochemotherapeutics can potentially be enhanced *in-vivo* through several molecular strategies that involve exploiting the over-expression of membrane HER2/*neu* and the molecular

properties of anti-HER2/*neu* as a selective "targeted" delivery platform. Given this perspective, endogenous trophic receptor over-expression is a critical variable that influences the cytotoxic anti-neoplastic potency of anthracycline-[anti-HER2/neu], anthracycline-[anti-EGFR] and related covalent immunochemotherapeutics because it provides opportunities to [*i*] significantly suppress neoplastic cell growth for populations with proliferation rates heavily dependent on tropic receptor over-expression; [*ii*] promote continual and selective chemotherapeutic deposition on the external surface membrane of neoplastic cells; and [*iii*] induce progressive active chemotherapeutic internalization by mechanisms of receptor-mediated endocytosis in a manner that promotes escalating increases in cytosol chemotherapeutic accumulation (Pimm, Paul, Ogumuyiwa, & Baldwin 1988; Shih et al., 1994; Stan et al., 1999; Yang et al., 1988a). The latter consideration is important since receptor-mediated-endocytosis of epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] and epirubicin-(C_3-*amide*)-SS-[anti-HER2/*neu*] can lead to increases in cytosol anthracycline concentrations that are 8.5x (Stan et al., 1999) to >100x (Pimm et al., 1988) greater than those that are attainable by simple passive anthracycline diffusion from the plasma or extracellular fluid compartments (e.g. following intravenous injection). Although specific data for HER2/*neu* and EGFR receptor complexes in chemotherapeutic-resistant mammary adenocarcinoma (SKBr-3) is limited, metastatic multiple myeloma internalizes approximately 8×10^6 anti-CD74 monoclonal antibody molecules per day following binding to membrane CD74 sites (Hansen, Ong & Diril 1996). Following selective "targeted" delivery more than 50% of an anthracycline at 24-hours is retained intracellularly (Stan et al., 1999) where it is primarily associated with either internal membrane structures or it becomes distributed throughout the cytosol environment (Liu et al., 2010; Shih et al., 1994). Conversely, "free" non-conjugated anthracycline upon passive diffusion across intact cellular lipid bilayer membranes is detected predominately in complex with nuclear DNA less than 30 minutes following initial exposure (Shih et al., 1994) whereas anthracycline liberated from covalent immunochemotherapeutics ultimately distributes into, and accumulates within the nucleus, mitochondria and golgi apparatus (Beyer, Rothen-Rutishauser, Unger, Wunderli-Allenspach & Kratz 2001).

Covalent immunochemotherapeutics like epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] and epirubicin (C_3-*amide*)-SS-[anti-HER2/*neu*] that posssess properties that include selective "targeted" delivery due to their binding avidity of anti-HER2/*neu*, anti-EGFR, anti-VEGR or other anti-trophic receptor monoclonal immunoglobulins also afford a therapeutic strategy for achieving additive and synergistic levels of cytotoxic anti-neoplastic potency. More specifically, their molecular design makes possible the simultaneous inhibition of over-expressed trophic receptor complexes in concert with the selective "targeted" delivery of conventional and non-conventional chemotherapeutics. Chemotherapeutic examples relevant anti-HER2/*neu* include cyclophosphamide, docetaxel, doxorubicin, etoposide, methotrexate, paclitaxel, and vinblastine (Pegram, Lopez, Konecny & Slamon 2000; Slamon et al., 2001). Similar to anti-HER2/*neu* (Boone, Bhosle, Tilby, Hartley & Hochhauser 2009; Meden, Beneke, Hesse, Novophashenny, & Wischnewsky 2001; Pegram et al., 2000; Slamon, & Pegram 2001; Slamon et al., 2001; Winer & Burstein 2001), anti-EGFR (Ciardiello et al., 1999; Kim et al., 2006; Landriscina et al., 2010) and anti-VEGF (García-Sáenz et al., 2008; Lynn et al., 2010; Zhang et al., 2002) can also evoke additive and synergistic levels of cytotoxic anti-neoplastic potency when applied in dual combination with conventional chemotherapeutics and other anti-cancer modalities. Variations in biological characteristics between and within different types of neoplastic cell populations likely accounts for differential planes of cytotoxic anti-neoplatic potency achieved with individual covalent immunochemotherapeutics and other forms of anti-cancer therapy. However, the potential opportunities that exist with many if not most covalent immunochemotherapeutics to achieve additive or synergistic levels of efficacy represent a distinctly attractive therapeutic advantage.

Conceptually, there are at least five variables that can be modified to achieve higher total cytotoxic anti-neoplastic potency levels for epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] and epirubicin-(C_3-*amide*)-SS-[anti-HER2/*neu*]. *First*, incubation times with mammary adenocarcinoma (SKBr-3) can be lengthened to periods >72 hours (Coyne et al., 2011) thereby allowing greater opportunity for larger amounts of epirubicin to be internalized by receptor-mediated-endocytosis and subsequent intracellular accumulation before and after liberation of the anthracycline moiety from epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] and epirubicin-(C_3-*amide*) -SS-[anti-HER2/*neu*].

Second, cytotoxic anti-neoplastic potency can be evaluated utilizing neoplastic cell types that are not classified as chemotherapeutic-resistant analogous to those applied in majority of the investigations published to date that describe the efficacy of related covalent immunochemotherapeutics (Figures 3 thru 10). Few anthracycline-immunoconjugates have been reported to exert cytotoxic anti-neoplastic potency against chemotherapeutic (multi-drug) resistant neoplastic cell populations that is relatively greater than the same anthracycline in a "free" unbound form (Dillman et al., 1989). Relevant exceptions are; [*i*] covalent epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] (Coyne et al., 2009), epirubicin-(C_3-*amide*)-[anti-EGFR] (Coyne et al., 2009) and

epirubicin-(C_{13}-*imino*)-[anti-HER2/neu] (Coyne et al., 2011b), against chemotherapeutic-resistant mammary adenocarcinoma (SKBr-3); [*ii*] covalent anthracycline ligand-chemotherapeutics utilizing epidermal growth factor (EGF) or an EDF-fragment against chemotherapeutic-resistant mammary carcinoma MCF-7AdrR (Lutsenko Feldman & Severin 2002); and [*iii*] covalent daunorubicin immunochemotherapeutic synthesized using anti-chondroitin sulfate proteoglycan 9.2.27 surface marker evaluated for cytotoxic anti-neoplastic potency against the metastatic melanoma M21 cell type (Dillman et al., 1989; Yang et al., 1988a; Yang & Reisfeld 1988b).

Third, cytotoxic anti-neoplastic potency of covalent immunochemotherapeutics can be assessed by measuring cellular proliferation with either $[H^3]$-thymidine, or an ATP-based assay method because of their reportedly ≥10-fold greater sensitivity in detecting early cell injury compared to MTT vitality stain based assay methods (Mueller, Kassack, & Wiese 2004; Ulukaya, Ozdikicioglu, Oral, & Dermirci 2008). Despite this consideration, MTT vitality stain continues to be extensively applied for the routine assessment of cytotoxic anti-neoplastic potency of chemotherapeutic agents in part because the detection of true cancer cell death is generally considered superior to the detection of reversible cellular injury (Dery, Van Themsche, Provencher, Mes-Masson, & Asselin 2007; Huang, Pierstorff, Osawa, & Ho 2007; Kars, Iseri, Gunduz, & Molnar 2008; Spee et al., 2006; Varache-Lembège, Larrouture, Montaudon, Robert, & Nuhrich 2008)

Fourth, cytotoxic anti-neoplastic potency can be delineated *in-vivo* utilizing human neoplastic xenographs in animal hosts as a neoplastic disease model where the efficacy of covalent immunochemotherapeutics frequently tends to be higher than in *ex-vivo* tissue culture models utilizing the same identical cancer cell type (Aboud-Pirak, Hurwitz, Bellot, Schlessinger, & Sela 1989; Johnson et al., 1995; Zhang, Wang, Li, Liu, & Dong 1992). Enhanced levels of covalent immunochemotherapeutic potency measured *in-vivo* that can not effectively be assessed in *ex-vivo* tissue culture is presumed to at least in part be dependent upon responses by the endogenous immune system through processes that include antibody-dependent cell cytotoxicity (ADCC) phenomenon in concert with complemented-mediated cytolysis activated by the formation of HER2/*neu*-immunoglobulin complexes on the exterior surface membrane of "targeted" neoplastic cells. Endogenous immune cell types involved in ADCC responses release cytotoxic mediators known to additively and synergistically enhance the cytotoxic anti-neoplastic activity of conventional chemotherapeutics (Coyne, Fenwick & Ainsworth 1997). The contributions of ADCC and complement-mediated cytolysis to the *in-vivo* cytotoxic anti-neoplastic potency of covalent anthracycline immunochemotherapeutics would be further complemented by the additive and synergistic properties attained with monoclonal immunoglobulin inhibitors of trophic receptors applied in dual combination with conventional chemotherapeutics (Ciardiello et al., 1999; Fry, Schilke, McGuire, & Bird 2010; García-Sáenz et al., 2008; Jin et al., 2010; Kim et al., 2006; Landriscina, Maddalena et al., 2010; Lynn et al., 2010; Pegram, Lopez, Konecny, & Slamon 2000; Slamon et al., 2001; Slamon & Pegram 2001; Winer & Burstein 2001; Zhang et al., 2002). Additive or synergistic interactions of this type have been detected with anti-HER2/*neu* in concert with cyclophosphamide, docetaxel, doxorubicin, etoposide, methotrexate, paclitaxel, or vinblastine (Pegram, Lopez, Konecny, & Slamon 2000; Slamon et al., 2001).

Fifth, the synthesis strategy for epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] and epirubicin-(C_3-*amide*)-SS-[anti-HER2/*neu*] could have been modified to increase the anthracycline molar-incorporation-index. Unfortunately, such modifications usually entail harsher synthesis conditions that impose a higher risk for declines in retained biological function and substantial reductions in final/total yield (Greenfield et al., 1990; Zhang et al., 1992). In addition to harsher synthesis conditions, excessively high molar incorporation indexes for anthracycline can (as previously discussed) also reduce the biological integrity of immunoglobulin fractions when the number of chemotherapeutic moieties covalently introduced into the Fab' antigen-binding region becomes excessive. Such modifications can result in only modest declines in immunoreactivity (e.g. 86% for a 73:1 ratio) but disproportionate declines in anti-neoplastic potency with reductions in activity to levels that are substantially lower than those associated with non-conjugated "free" anthracycline (Zhang et al., 1992).

4.4 Cytotoxic Anti-Neoplastic Potency of Benzimidazoles

The benzimidazole anthelmintics exert a mechanism-of-action in neoplastic cells that is distinctly different, but similar to that of the vinca alkaloids (Spagnuolo et al., 2010) which involves binding to colchicine-sensitive sites on β-tubulin protein. Due to this mechanism-of-action, the benzimidazoles inhibit tubulin polymerization or tubulin de-polymerization resulting in a suppression of normal microtubule assembly and function necessary for mitosis (cell cycle M-phase). Coincident with a disruption of mitosis, benzimidazole tubulin/microtubule inhibitors are believed to induce apoptosis in neoplastic cells through a variety of pathways based upon detected elevations in Bcl-2 phosphorylation, caspase-3, caspase-8, caspase-9, cytochrome-C release, p53, DNA laddering phenomenon, and DNA fragmentation (TUNEL) (Doudican, Rodriguez, Osman, & Orlow 2008; Khalilzadeh,

Wangoo, Morris, & Pourgholami 2007; Martarelli, Pompei, Baldi, & Mazzoni 2008; Pourgholami, Akhter, Wang, Lu, & Morris 2005; Sasaki et al., 2002). Declines in neoplastic cell growth and vitality induced by benzimidazole tubulin/microtubule inhibitors have been recognized as a function of alterations in parameters that reflect G_2/M and G_0-G_1 arrest, decreased [^{3}H]thymidine incorporation, spheroid cell formation, altered cell vitality staining intensity, and lower functional growth characteristics (Pourgholami et al., 2005; 2001; Sasaki et al., 2002; Martarelli et al., 2008). In neoplastic tissues, benzimidazole anthelmintics have been shown to reduce expression of CD31 (tumor angiogenesis biomarker), carcinoembryonic antigen (CEA: *in-vivo*), and α-feto protein (AFP: *in-vivo*); while also suppressing migration/invasion (*in-vitro*), metastasis (*in-vivo*), and tumor (*in-vivo*) growth kinetics (Martarelli et al., 2008; Mukhopadhyay, Sasaki, Ramesh & Roth 2002; Morris, Jourdan & Pourgholami 2001). Preliminary experimental investigations have detected adrenocortical carcinoma (xenographs), colorectal cancer, hepatocellular carcinoma, leukemia, lung cancer, (non-small cell), melanoma (chemo-resistant), myeloma, and ovarian cancer that are sensitive to benzimidazole tubulin/microtubule inhibitors (Martarelli et al., 2008; Morris, Jourdan & Pourgholami 2001; Khalilzadeh et al., 2007; Spagnuolo et al., 2010; Mukhopadhyay et al., 2002; Sasaki et al., 2002; Doudican et al., 2008; Pourgholami et al., 2001; 2005; 2009; 2010). The cytotoxic anti-neoplastic potency of the benzimidazole class of tubulin/microtubule inhibitors against breast cancer has previously remained largely unknown.

In chemotherapeutic-resistant human mammary adenocarcinoma (SKBr-3) the benzimidazoles, albendazole, mebendazole and flubendazole all demonstrated a degree of cytotoxic anti-neoplastic potency at a final concentration range between 0-to-2.5 μM (Figure 5). Previous descriptions have reported very similar results against other neoplastic cell types (Doudican et al., 2008; Khalilzadeh et al., 2007; Martarelli et al., 2008; Pourgholami et al 2005; Pourgholami et al., 2001; Sasaki et al., 2002; Spagnuolo et al., 2010). Flubendazole was the most potent benzimidazole while albendazole was substantially less potent than either flubendazole or mebendazole which closely correlates with their relative order of cytotoxic anti-neoplastic potency against leukemia and myeloma cell types (Figure 5) (Spagnuolo et al., 2010). In contrast to flubendazole, the creation of mammalian chromosomal aberrations has to date not been reported for either albendazole or mebendazole (Nianjun, Cerepnalkoski, Nwankwo, Dews, & Landolph 1994).

4.5 Cytotoxic Anti-Neoplastic Potency of Griseofulvin

The anti-fungal tubulin/microtubule inhibitor, griseofulfin and the organoselenium compound methylselenocysteine both exerted cytotoxic anti-neoplastic potency against mammary adenocarcinoma (SKBr-3) when formulated at final concentrations between 0-to-100 μM (Figure 6). Various organoselenium compounds including methylselenocysteine are known to exert cytotoxic anti-neoplastic properties against mammary adenocarcinoma/carcinoma (Coyne et al., 2011; Ip & Dong 2001; Johnson, Morrissey, Kapetanovic, Crowell, & McCormick 2008; Li et al., 2009; Medina, Thompson, Ganther, & Ip 2001) and other cancer cell types (Cao, Durrani, & Rustum 2004; Chintala et al., 2010) while also enhancing the potency of anthracyclines (Coyne et al., 2011; Juliger, Goenaga-Infante, Lister, Fitzgibbon, & Joel 2007; Li, Zhou, Dong & Ip C 2007; Li, Zhou, Wang, Zhang, Dong, & Ip 2007) and covalent epirubicin-immunochemotherapeutics (Coyne et al., 2011). Somewhat surprisingly the cytotoxic anti-neoplastic potency of griseofulvin against mammary adenocarcinoma (SKBr-3) was substantially greater than molar-equivalent (standardized) concentrations of methylselenocysteine (Figure 6). The final concentrations of 20 and 40 μM most prominently reflected this property but the difference was marked at 20 μM (Figure 7).

Similar to the benzimidazole tubulin/microtubule inhibitors, the mechanism-of-action for griseofulvin involves binding to tubulin protein and disruption of microtubule function resulting in an inhibition of normal mitosis (Rathinasamy et al., 2010). In neoplastic cells griseofulvin also promotes inhibition of centriole clustering, stabilization of microtubule dynamics, and G_2/M arrest (Rathinasamy et al., 2010; Ho et al., 2001; Uen, Liu, Weng, Ho, & Lin, 2007). Failure of chromosomal division in turn induces tumor cell death but interestingly does not detectably influence normal healthy cell populations. Griseofulvin additionally stimulates p53 activation and induces apoptosis reflected by the detection of increases in DNA fragmentation ("laddering"), nuclear lamin alterations, accompanied by induced alterations in expression profiles for Cdc2 kinase, caspase-8, caspase-9, and changes in viability staining characteristics (Rathinasamy et al., 2010; Ho et al., 2001; Uen et al., 2007). Neoplastic cell types that clinical may be sensitive to griseofulvin include mammary carcinoma, cervical carcinoma, colorectal carcinoma, oral squamous cell carcinoma, hepatocellular carcinoma, osteosarcoma and myeloid leukemia (Panda, Rathinasamy, Santra, & Wilson, 2005; Rebacz et al., 2007; Ghadimi et al., 2000; Rønnest et al., 2009; Rathinasamy et al., 2010; Ho et al., 2001; Uen et al., 2007).

The benzimidazole anthelmintics and griseofulvin anti-fungal agent have a mechanism-of-action that is highly analogous to that of many conventional tubulin/microtubule inhibitor chemotherapeutics. Even though very little

is known about the cytotoxic anti-neoplastic properties of benzimidazole anthelmintic and griseofulvin anti-fungal agents, their mechanism-of-action suggests that they can potentially suppress the growth and vitality of cancer cell populations both alone, and in multi-chemotherapeutic regimens similar to the vinca alkaloids, taxanes (e.g. paclitaxel), podophyllotoxins (e.g. etoposide) and monomethyl auristatin E (MMAE). The cytotoxic anti-neoplastic potency of epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] and epirubicin-(C_3-*amide*)-SS-[anti-HER2/*neu*] formulated between the final epirubin-equivalent concentrations of 10^{-10} M to 10^{-6} M was markedly increased when applied in concert with mebendazole and griseofulvin (Figures 7 & 8). Mebendazole and griseofulvin evoked slightly higher levels of cytotoxic anti-neoplastic activity in dual combination with epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] than was detected with epirubicin-(C_3-*amide*)-SS-[anti-HER2/*neu*] but the variable responsible for this trend remains unknown although it may possibly be related to variations in intracellular epirubicin bioavailability or differences in the lipophilic characteristics of different epirubicin metabolite analogs (Figures 7, 8, 9 & 10) (Erickson et al., 2010)**.**

Capacity of the benzimidazole class of tubulin/microtubule inhibitors to evoke additive or synergistic levels of cytotoxic anti-neoplastic potency when applied in dual combination with conventional (e.g. vinblastine) (Spagnuolo et al., 2010) or selectively "targeted"/delivered chemotherapeutic agents has previously been very rarely delineated. Similarly, the potential for griseofulvin in combination conventional chemotherapeutics in multi-combination regimens to create additive or synergistic levels of cytotoxic anti-neoplastic potency has to date been investigated on a very limited scale. In preliminary investigatins, however, griseofulvin has been found to complement the anti-neoplastic properties of nocodazole (Ghadimi et al., 2000; Ho et al., 2001) and vinblastine (Rathinasamy et al., 2010).

Despite these voids in knowledge, multiple implications arise upon the acknowledgement that benzimidazole and griseofulvin tubulin/microtubule inhibitors can produce additive or synergistic cytotoxic anti-neoplastic potency when applied in dual combination with conventional or selectively "targeted" chemotherapeutics. Results observed with the benzimidazoles and griseofulvin tubulin/microtubule inhibitors indicate that they potentially have realistic utility as an alternative class of chemotherapeutic capable of providing "new" opportunities for achieving more potent long-term resolution of even the most resistant forms of breast cancer and other neoplastic disease states while simultaneously posing a lower risk of undesirable sequelae. In either a mono-therapy format or as a component of a combination multi-chemotherapeutic regimen these attributes can potentially be attained because benzimidazole and griseofulvin tubulin/microtubule inhibitors are apparently poor P-glycoprotein substrates (Khalilzadeh et al., 2007; Spagnuolo et al., 2010) and they have a relatively wider margin-of-safety than many if not most conventional chemotherapeutics (de Silva, Guyatt & Bundy 1997; Morris et al., 2001b; Pourgholami et al., 2005; 2010). The benzimidazole anthelmintics and griseofulvin when applied in additive and synergistic combinations with other chemotherapeutics or anti-cancer modalities can potentially add another level of safety because they are able to ultimately afford lower total dosage requirements. Presumably the highest levels of additive and synergistic cytotoxic anti-neoplastic potency and widest margin-of-safety can be attained when benzimidazole anthelmintics or griseofulvin are substituted for conventional tubulin/microtubule inhibitor chemotherapeutics in combination regimens that also apply covalent anthracyline-immunochemotherapeutics with properties of selective "targeted" delivery. Such considerations are critically important to the development of safer and more effective treatment regimens in order to reduce collateral cardiotoxicity (Danesi et al., 2006; Last et al., 2003; Nakano, Takeshige, Toshima, Tokunaga, & Minakami 1989) and nephroticity (Bulucu et al., 2008) that commonly limit systemic anthracycline administration.

5. Conclusion

Cardiotoxicity (doxorubicin >> epirubicin) (Danesi et al., 2006; Last et al., 2003; Nakano et al., 1989), nephroticity (Bulucu et al., 2008) and chemotherapeutic resistance represent complications that can commonly limit anthracycline administration in modern clinical oncology. The molecular design and methodology delineated for the synthetic production of covalent epirubicin-immunochemotherapeutics utilizing a UV-photoactivated anthracycline intermediate addresses a need to discover and optimize laboratory methods for the expedient production of anti-cancer therapies at higher end-product yields that possess properties of selective "targeted" delivery that complement the efficacy and potency of conventional and unconventional chemotherapeutics. In this context, selective "targeted" epirubicin delivery affords the opportunity to achieve cytosol concentrations that are greater than can be attained by simple passive diffusion, serve as a molecular mechanism for minimizing the impact of chemotherapeutic-resistance in many forms of neoplastic disease, and it serves as a way of minimizing chemotherapeutic diffusion into innocent tissues and organ systems (potential for a relatively wider margin-of-safety). Covalent anthracycline immunochemotherapeutics are relevant to the treatment of breast

cancer and many other neoplastic conditions complicated by aggressive localized growth characteristics or have a high probability for metastasis and therapeutic resistance (Alexander, Greene, Torti & Kucera 2005). Preliminary laboratory analyses that detects and measures over-expression of trophic membrane receptors (e.g. HER2/neu, EGFR, VEGFR), proteins associated with chemotherapeutic resistance (e.g. P-glycorprotein, breast cancer type susceptibility protein/BRCA1 (Chekhun et al., 2009), and endocrine receptor profiles (e.g. estrogen, progesterone, testosterone) could be applied to account for biological variations and identify neoplastic conditions most effectively resolved with covalent immunochemotherapeutics like epirubicin-(C_3-*amide*)-[anti-HER2/*neu*]. Base on these considerations, future investigations devoted to delineating the benefit of implementing epirubicin-(C_3-*amide*)-[anti-HER2/*neu*] in the formulation of individualized treatment protocols is warranted.

Discovery of the cytotoxic anti-neoplastic potency of the benzimidazole anthelmintics and griseofulvin against mammary adenocarcinoma (SKBr-3) illustrates their potential applicability as an alternative class of tubulin/microtubule inhibitor chemotherapeutics and encourages support for future investigations devoted to determining their role in the development of new treatment regimens. Relevant attributes include a greater level of efficacy against certain chemotherapeutic-resistant neoplasias that over-express P-glycoprotein and the opportunity they provide to additively or synergistically complement the efficacy of conventional and selectively "targeted"/delivered chemotherapeutics. Such considerations are directly relevant to current trends and objectives in modern clinical oncology directed at identifying multi-treatment protocols with a wider margin-of-safety that more potently resolve locally aggressive, highly metastatic and resistant forms of cancer.

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Stereotactic Brain Biopsy or Bronchoscopic/Transthoracic Needle Biopsy for Diagnosis of Metastatic Cancer Presenting Simultaneously in Lung and Brain: A Comparison of Safety and Efficacy

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Abstract

Background: When patients present with simultaneous lung and brain lesions consistent with metastases, it is often presumed that it is safer and less invasive to biopsy the lung lesion. **Objective:** To determine whether lung biopsy or stereotactic brain biopsy has a higher diagnostic yield and lower morbidity for tissue diagnosis in patients with simultaneous brain and lung lesions. **Methods:** Retrospective review of the author's stereotactic biopsy series and of the literature on brain and lung biopsies for suspected malignancy. **Results:** The overall diagnostic yield for bronchoscopic lung biopsy ranged from 44% to 88% and the pneumothorax rate from 1.2% to 8%. No deaths were reported. The overall diagnostic yield for transthoracic lung biopsy ranged from 74% to 96% and pneumothorax rate from 2.2% to 8%. No deaths were reported. The overall diagnostic yield for stereotactic brain biopsy ranged from 90.6% to 99.3% when all potential diagnoses are included. Complication rates ranged from 0.6% to 4.8% with mortality from 0% to 1.5%. Several series reported no mortality. **Conclusion:** Stereotactic brain biopsy has a higher diagnostic yield and a lower complication rate, but a higher mortality. The inclusion of diagnoses other than metastases in the reported series may account for some of the reported mortality. When lung and brain lesions are detected simultaneously, stereotactic biopsy is a better option for tissue diagnosis.

Keywords: stereotactic biopsy, metastasis to brain, lung biopsy

1. Introduction

Metastasis to brain is often the first presentation of lung and other malignancies. Once the brain lesion(s) is detected, a search for the primary usually ensues. Computed tomography (CT) of the chest, abdomen, and pelvis is often the next diagnostic test. If a lesion(s) is detected in the lung, then bronchoscopic or transthoracic biopsy is usually attempted to establish a tissue diagnosis and to plan subsequent treatment (Dasgupta & Mehta, 1999). The presumption has been that this is safer and easier than a biopsy of the brain lesion. However, the safety of stereotactic needle biopsy of brain lesions is now well established. The incision required is small and discomfort is minimal; no hair need be shaved (Sheinberg & Ross, 1999); and some studies report that this can safely be done as an outpatient (Bhardwaj & Bernstein, 2002; Kaakaji et al., 2001).

2. Method

The author reviewed his neurosurgical practice (Michigan and Oregon, USA) stereotactic biopsy series for malignant tumors of the brain and conducted a Medline literature search to compare the safety and efficacy of stereotactic biopsy with the reported safety and efficacy of bronchoscopic and transthoracic lung biopsy. The goal was to learn which is actually the safer and higher yielding procedure.

3. Results

From 1989 to 2009, the author conducted 161 (78 female/83 male; mean age 51.5 years) framed based or frameless stereotactic brain biopsies for malignant tumors of the brain. One hundred and forty-seven were for gliomas (106), central nervous system lymphomas (31), or pineal tumors and other rare primary central nervous system pathologies (10). Fourteen were for the tissue diagnosis of metastases to the brain. During this same time period, 150 resective procedures were performed for brain metastases. Three procedures (1.9%), two for suspected glioma and one for primary central nervous system lymphoma were initially nondiagnostic and had to be repeated, following which one remained nondiagnostic (0.6%). There was a single complication of a minor

intraventricular hemorrhage following biopsy of a glioblastoma in the genu of the corpus callosum which resolved without surgery or new neurologic deficit, but prolonged the hospitalization (0.6%). There were no new neurological deficits, no infections, and no deaths.

4. Discussion

4.1 Bronchoscopic Biopsy

There are variations in how diagnostic bronchoscopic biopsy is performed and reasons for selecting this technique over transthoracic needle biopsy. For a review of this topic, see Yung (2003). In one study, the diagnostic yield of transbronchial lung biopsy with fluoroscopic guidance was reported to be 43.8% with an incidence of pneumothorax of 1.2% (Rittirak & Sompradeekul, 2007). When endobronchial ultrasound, electromagnetic navigation, or both were used without fluoroscopy, diagnostic yields of 69%, 59%, or 88% have been reported (Eberhardt, Anantham, Ernst, Feller-Kopman, & Herth, 2007). The pneumothorax rate was 5-8% with these procedures (Eberhardt, et al., 2007). CT guidance with virtual bronchoscopy and an ultrathin endoscope for peripheral lung lesions yielded a diagnosis in 65.4% of patients (Shinagawa et al., 2004). Thus, diagnostic yield ranged from 44% to 88% and the pneumothorax rate from 1.2% to 8%. No deaths were reported.

4.2 Transthoracic Biopsy

For 46 patients with lesions near the chest wall, diagnostic yield of ultrasound guided transthoracic biopsy was 95.6% with one case each of hemoptysis (2.2%) and one pneumothorax (2.2%) (Seyfarth et al., 2007). In another study of 91 patients undergoing ultrasound guided biopsy, the diagnostic sensitivity was 85.5% and the risk of pneumothorax 4% (Diacon et al., 2004). CT guided biopsy in 147 cases yielded a diagnostic accuracy of 94.6% with pneumothoraces in 12.9% of which 2.7% required a chest tube (Kinoshita et al., 2006). In 506 consecutive patients, pneumothorax was detected immediately in 22.9%, treated immediately in 6.5%, and detected and treated in a delayed fashion in another 1.4% (Dennie, Matzinger, Marriner, & Maziak, 2001). In 343 biopsies performed in patients most of whom had non-diagnostic bronchoscopy, the diagnosis was made in 73.7% of malignant lesions, with pneumothorax in 7.7%, of whom 1.6% needed a chest tube, and hemorrhage in 1.3% (Schneider et al., 1999). Thus, diagnostic yield ranged from 74% to 96% and pneumothorax from 2.2% to 8%. No deaths were reported. Complications have been reported to be related to multiple punctures, longer intraparenchymal needle tract, and smaller lesion size (Nakatani et al., 2012; Smayra et al., 2012).

A recent economic analysis comparing CT guided needle biopsy to ultrasound guided transbronchial biopsy found the two modalities to be equivalent overall, but found that in specific cases one maybe more effective than the other (Steinfort, Liew, & Irving, 2012).

4.3 Stereotactic Brain Biopsy

It is difficult to discern from the published literature on stereotactic brain biopsy how many procedures were performed for malignancies, either primary or secondary. A recent review of 290 cases of CT guided biopsy reported a diagnostic biopsy in 95.5%, a 4.1% incidence of symptomatic hemorrhage (two required surgery), and a mortality of 0.8% (Ersahin et al., 2011). A recent series of 134 patients done without frozen section confirmation had a diagnostic yield of 99.3% (one targeting error) and complications in 2.2%, one of which was a conservatively treated hematoma and two of which were fatal in high grade gliomas (1.5%) (Shooman, Belli, & Grundy, 2010). In a series of 299 biopsies by 11 surgeons for all diagnoses, diagnostic yield was 90.6% with symptomatic hemorrhage in 4.4% and death in 1.3% (Chen et al., 2009). In another series of 622 diagnostic biopsies for all histologies, the diagnostic yield was 98.4%, the overall morbidity 6.9%, the symptomatic hemorrhage rate was 4.8%, new, persisting neurologic deficits occurred in 1.5% and death in 1.3% (Kongkham, Knifed, Tamber, & Bernstein, 2008). Complications were more likely in deep seated lesions and glioblastoma than other diagnoses. In 465 biopsies over a 10 year period, the diagnostic yield was 89.4% with symptomatic hemorrhage in 3.8% and mortality in 1.5% (Dammers et al., 2008). In this series, complications were more common in frontotemporal biopsies and when the diagnosis was lymphoma.

In a series of 270 patients, a symptomatic hematoma occurred in 5%, with a glucose level of greater than 200 highly associated with the likelihood of complications and thalamic or basal ganglia lesions at higher risk as well (McGirt et al., 2005). In a series of 355 biopsies, the diagnostic yield was 93.8% with a symptomatic hemorrhage rate of 3.6% and a mortality of 0.6%, with brainstem biopsy being the only factor associated with higher morbidity (Grossman, Sadetzki, Spiegelmann, & Ram, 2005). A series of 153 patients in whom a micro Doppler probe was used to look for vessels prior to biopsy yielded a diagnosis in 98%, a CT detected hemorrhage in 2.5%, permanent neurologic deficit in 0.6%, and no mortality (Hertel, Feiden, & Bettag, 2005). A series of 69 biopsies for inoperable lesions produced no morbidity and no mortality (Stranjalis, Protopapa, Sakas, & Chondros, 2003). In

two series from the same institution, 130 biopsies resulted in 3.8% symptomatic complications (4/5 transient) and one death (0.8%) and 138 biopsies resulted in 2.2% symptomatic complications (one of which was a hematoma) and no deaths (Kaakaji et al., 2001). In a series of 225 patients of which 12.9% harbored metastatic tumors, biopsy was diagnostic in 95.6%, major morbidity (hemorrhage or neurologic deficit) occurred in 3.6% and there was one death (0.4%) (Sawin, Hitchon, Follett, & Torner, 1998). Morbidity was linked to antiplatelet agents, deep seated lesion, chronic steroid use, and gliomas, but was not linked to extracranial malignancy. In a series of 122 patients by a single author (Hall, 1998), the diagnostic yield was 96% with 0.7% morbidity and one (0.7%) fatality. Hall reviewed the published literature to date in his 1998 paper, finding 7471 reported cases with an overall mortality of 0.7%, morbidity of 3.5%, and diagnostic yield of 91%. In another series of 500 biopsies, over 2000 specimens were obtained from 741 targets with a complication rate of 1% and mortality of 0.2% (Apuzzo, Chandrasoma, Cohen, Zee, & Zelman, 1987). The diagnostic yield overall ranged from 89.4% to 99.3% (95.6% overall) when all potential diagnoses were included. Non diagnostic biopsies did show necrosis in 10 cases, inflammatory response in nine cases, granuloma in one case, and gliosis in one case. On aggregate, complication rates for stereotactic brain biopsy for all pathologies ranged from 0.6% to 4.8% with mortality from 0% to 1.5%. Stereotactic brain biopsy results are summarized in Table 1.

Table 1. Summary of stereotactic brain biopsy results

Publication (First Author, Year)	Patients (n)	Diagnostic Yield (%)	Major Complication (%)	Death (%, (n))
Ross DA, 2012*	161	99.4	0.6	0, (0)
Shooman D, 2010	134	99.3	2.2	1.5, (2)
Chen C-C, 2009	299	90.6	4.4	1.0, (3)
Kongkham PN, 2008	622	98.4	6.9	1.3, (8)
Dammers R, 2008	465	89.4	3.8	1.5, (7)
Grossman R, 2005	355	93.8	3.6	0.6, (2)
Hertel F, 2005	153	98	2.5	0 (0)
Sawin PD, 1998	225	95.6	3.6	0.4, (1)
Hall WA, 1998	122	96	0.7	0.8, (1)
Apuzzo ML, 1987	500	95.6	1	0.2, (1)

*data reported here

Overall, in the published literature, when compared to lung biopsy, stereotactic brain biopsy had a higher diagnostic yield and a lower morbidity, but a higher mortality. Pneumothorax and hemoptysis were the most common complications of lung biopsy; whereas, new neurologic deficit, hematoma, or death were reported in brain biopsy, but it was not possible to discern if complications were more common in diagnoses other than metastatic tumors. In the author's hands, stereotactic biopsy had a higher yield, lower morbidity, and no mortality when compared to lung biopsy. Other authors have reported similar results (Apuzzo et al., 1987; Hertel et al., 2005; Shooman et al., 2010; Stranjalis et al., 2003). It is likely that the wide range of pathologies included in the other published series of stereotactic brain biopsy may contribute to the morbidity of these procedures. Glioblastomas (Kongkham et al., 2008; Sawin et al., 1998) and lymphomas (Dammers et al., 2008) have been reported as more likely to bleed when biopsied. Targets located adjacent to the ventricles or the Sylvian fissure may be especially challenging and biopsy of vascular anomalies or infarcts may be disastrous (Neal & Apuzzo, 1989). Since many metastatic tumors to the brain are located in the gray-white junction of the hemispheres, the thalamus, or the cerebellum and present straightforward targets for stereotactic biopsy, it may be that the morbidity of these procedures is lower than the overall reported morbidity for stereotactic biopsy, but this data cannot be gleaned from the literature.

In order to be less morbid than lung biopsy, stereotactic brain biopsy should be performed with no mortality and with rare serious complications. If these criteria can be met, whenever the lung mass is thought to be technically suboptimal for biopsy or if the brain mass is in an accessible location for stereotactic biopsy, the brain lesion should be considered for biopsy in preference to the lung lesion.

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The Cost of Cancer Care: Patient Navigation Challenges to Participation in Phase I Clinical Trials

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Abstract

Phase I trial participation for advanced cancer patients is often a "last resort" in an attempt to control progressive disease. It is also a necessary component mandated by our investigative community and FDA to characterize safety and mechanism via dose and schedule modulation of experimental therapeutics. Participation in phase I studies is difficult without support. The purpose of this assessment is to provide proof of principle mechanisms of support locally provided in Texas and established by nurse navigation to facilitate phase I trial patient participation. Specifically, extensive financial assistance programs exist to facilitate patient participation. Management of these opportunities through a nurse navigation program in coordination with the patient optimizes patient assistance.

Keywords: Navigator, cancer, cost, trial, phase I

Phase I clinical trials provide a unique opportunity to cancer patients who have failed standard of care (SOC) options, but the cost of treatment constitutes a significant patient concern. Patient Navigation programs are established to assist medical care management involving multi-specialty care and often include clinical research options (Ramsey et al., 2009; Thygesen et al., 2011). As Patient Navigation programs increase in prevalence, program models are evolving but often don't focus on the additional financial stress to the patient and family related to the more complicated care management required for clinical trials. This is particularly relevant to phase I clinical trial participation. While the sponsor of the clinical trial covers the cost of the study drug and research related expenses, other expenses are the responsibility of the patient or their insurance carriers. Uninsured patients have been shown less likely to participate in clinical trials (Sateren et al., 2002). However, insured patients also face financial obstacles to clinical trial participation. Some insurance plans do not cover standard non-research costs if patients participate in research. Medicare has a policy of covering standard costs for patients enrolled in research trials, and 29 states have enacted legislation mandating such coverage for all health insurance plans governed by their state (Klamerus et al., 2010). However, state laws do not impact plans governed by the federal Employee Retirement Income Security Act (ERISA). Certain employers can operate under a self-insurence scheme in which they are only governed by ERISA, and not by state law. This has proven a popular business decision to reduce costs for employers due to recent premium increases. On January 1, 2014, the minimum coverage mandated by the Patient Protection and Affordable Care Act will eliminate this loophole, and all plans will be required to cover non-research costs for patients enrolled in clinical trials (Klamerus et al., 2010). Until that time, clinical research centers must pre-certify all standard procedures before enrolling patients into a trial. Pre-certification can cause a delay of up to six weeks, a time period that can be fatal to a late stage cancer patient seeking to enroll on a trial and receive an investigational agent as a last hope, after standard therapies have failed.

Although insurance companies in most states are legally obligated to cover standard of care costs involved in clinical trials, coverage is limited by deductible and co-insurance amounts as well as benefit limits, which may have been exhausted by previous payment for standard of care treatments. Increasingly, managed care insurance plans limit the number of chemotherapy regimens allowed. The required precertification of all SOC costs, procedures and diagnostic imaging may create barriers to providers and patients from obtaining necessary payment for standard reimbursable costs. During precertification, it is essential to distinguish between SOC and

research costs and to follow insurance companies' guidelines, which often prove difficult to determine and variable in their application by insurance company staff. Failure to precertify may result in the loss of benefits, leaving the patient or the provider with an unpaid balance.

In the current economic climate Oncology Nurse Navigators can play an integral part in health care reform and cost containment. Addressing barriers to care leads to better compliance, potentially less treatment, improved quality of life, and earlier detection of complications – all of which save precious health care dollars (Beerman, 2010).

Clinical trial centers need processes to help individuals with financial assistance, and Patient Navigation programs can be designed to interface with the finance team. The team can include patient Financial Counselors to verify insurance coverage, discover coverage gaps, and explain the patient's financial responsibility before treatment begins. Patient consent should outline the patient's responsibility for SOC costs, including drugs, imaging, labs, so they are informed of this during the consent process. Patients should be given their estimated financial responsibility for a trial, up front. This will require an estimate of pre-certified insurance coverage and co-pay responsibility. Patient Navigators can identify additional financial needs, which include lodging and transportation as well as co-pays for treatment and diagnostic testing, and payment for supportive medications. For patients covered by 70/30 or 80/20 insurance plans, study-related SOC drug expense may represent a significant financial burden. Patient Navigation programs should incorporate communication of financial assistance program information at the time of consent for treatment. Identification of financial assistance programs may determine whether or not the patient is able to participate in a clinical trial.

Numerous well-documented barriers contribute to the under representation of lower income individuals in clinical trials. These include lack of adequate knowledge about available studies and lack of adequate insurance to allow participation. Cultural factors contribute to fears of cancer research and impede communication between patients and health care providers. Residential distance from the centers where research is conducted presents logistical barriers. Addressing logistical challenges such as transportation, child care, and difficult clinic scheduling can help to make clinical trials accessible to a wider demographic population (Holmes et al., 2012). Patient Navigation programs can be structured to guide and support patients through all of these logistical challenges.

Financial assistance programs include co-pay assistance, drug assistance or replacement programs by pharmaceutical companies for SOC drugs needed on a trial, but not covered by the research sponsor. For example, organizations providing financial assistance with medications include: Rx Assist; Patient Advocate Foundation Co-Pay Relief; NeedyMeds; and Partnership for Prescription Assistance. Other organizations assisting with patient's financial and emotional needs include Cancer Care, which provides one time payments for any patient need including medication, transportation or living expenses, supported in part by a grant from the Hirshberg Foundation; and Patient Advocate Foundation. See Table 1 for website and phone numbers for these organizations and others discussed below.

Table 1. Patient assistance organizations

Name of Organization	Website Address	Phone Number
Rx Assist	www.rxassist.org	401-729-3284
Patient Advocate Foundation Co-Pay Relief	www.copays.org	866-512-3861
NeedyMeds	www.needymeds.org	978-281-6666
Partnership for Prescription Assistance	www.pparx.org	888-477-2669
Cancer Care	www.cancercare.org	800-813-4673
Patient Advocate Foundation	www.patientadvocatefoundation.org	800-532-5274
Bridge of Blessings	www.bridgeofblessings.org	214-714-1077
National Pancreatic Cancer Foundation	http://pancreaticcancerfoundation.com/	800-859-6723
Susan G. Komen for the Cure	http://ww5.komen.org/	877-465-6636
Careity® Foundation	www.careity.org	817-882-4100
American Cancer Society	www.cancer.org	800-227-2345
Cancer Care Services	www.cancercareservices.org	817-921-0653
Carcer Care, Inc.	www.carecare.org	800-813-4673
Lance Armstrong Foundation	www.livestrong.org	877-236-8820
Kids Konnected	http://kidskonnected.org/	800-899-2866
Angel Flight	www.angelflight.com	888-426-2643
Wings of Hope	www.wings-of-hope.org	800-448-9487
Mary Crowley* Benevolence Program	www.marycrowley.org	866-902-2623
Ark House	www.arkhouse.net	972-671-7144
Meals On Wheels, Inc. of Tarrant County	www.mealsonwheels.org	817-336-0912

* Applicable only for Mary Crowley study patient participants

In addition, disease-specific organizations exist, for example, the Bridge Breast Network in Dallas, Texas, serves uninsured and underinsured women with breast cancer. Another example is Bridge of Blessings in Richardson, Texas, which helps breast and ovarian patients with rent, utilities, and gas. The National Pancreatic Cancer Foundation benefits patients with a pancreatic cancer diagnosis.

Patient Navigators should keep current with all local programs that serve cancer patients in their vicinity. This includes local hospital/clinic general patient assistance programs. Each year the Cancer Assistance Fund of North Texas provides financial assistance to help with a portion of COBRA payments. A limited amount of funds are available to assist cancer patients in Tarrant, Johnson, Wise and Parker counties who are in serious financial need with the cost of their cancer treatment programs. For those patients in financial need with minimal (e.g., Medicaid) or no health insurance the fund may be able to make direct payments to oncology care providers. Support is provided by generous gifts from the Susan G. Komen Greater Fort Worth Affiliate and Careity® Foundation.

The American Cancer Society provides education services and serves as a resource for identifying community assistance. Though ACS is limited in the amount of funds provided to each individual, programs assist with lodging and gasoline expenses. Many patients receive vouchers for travel expenses. The Road to Recovery program depends on volunteers to provide transportation to treatment. Other examples of agencies providing assistance with transportation costs include Cancer Care Services, which offers travel and prescription assistance for patients receiving radiation. Cancer Care, Inc. in New York can help with a one-time grant of $100 for transportation.

The Lance Armstrong Foundation offers a wide range of services, including Oncology Nurse Navigators who provide education information, as well as assistance in identifying resources for financial assistance. Licensed professional counselors are available free of charge for patients and family members dealing with a cancer diagnosis.

Kids Konnected offers free programs for children that have a parent with cancer and may be a good resource for kids especially for emotional support.

Nonprofit charitable organizations of pilots, volunteers, and friends provide air transportation for medically related needs. One such organization, Angel Flight, primarily services patients needing transportation to or from the heartland region. However, by coordinating with other organizations, they may arrange transportation for patients on longer flights or to other parts of the country. Angel Flight is supported primarily by volunteer pilots who fly the missions and donate the use of their airplanes and operating expenses, and by contributions from individuals, service clubs, social and religious groups and corporations. In 2003, Wings of Hope established the St. Louis based Medical Relief and Air Transport Program, referred to as the MAT. Volunteer pilot and volunteers provide medical transportation free of charge.

Clinical Trial Centers may use philanthropy to maintain institution-specific financial assistance programs. For example, the Mary Crowley Benevolence Program was established to provide assistance with travel and medical expenses, including flights, gas, lodging, prescriptions, imaging and other underinsured medical expenses for patients participating in clinical trials at Mary Crowley Cancer Research Centers (Mary Crowley) in Dallas, Texas. The Mary Crowley Patient Navigation Program in collaboration with Medical City Dallas Hospital and Baylor Regional Medical Center at Plano provides cancer patients a continuum of care including early phase clinical trials, with program design including financial aspects as well as multi-disciplinary medical care.

Some phase I clinical trials require the patient to be present for treatment or follow-up on a weekly or daily basis. Additional out-of-pocket costs related to travel to the phase I study site, housing at the site of treatment, combined with time off work lead many patients to hesitate when considering phase I clinical trial participation. At Mary Crowley, the Patient Navigation Program has incorporated several sources of assistance to reduce patient travel and lodging burden. For patients who live outside the clinical research center's area, lodging can be a challenge. One excellent resource available in Dallas, Texas, is the Ark House. A local church leases twenty units at an apartment complex near the Mary Crowley clinic, and subleases the units to families requiring lodging for more than seven days. Each one bedroom unit is fully furnished with everything except toiletries and food. Patients pay a refundable deposit of $100 and $15 per night, and can stay as long as they continue medical treatment.

Several clinical trial sponsors (pharmaceutical corporations) offer reimbursement for lodging to patients enrolled in specific studies. When this option is available, Mary Crowley has established direct billing to reduce the patient burden. In situations where the sponsor does not cover the cost of lodging it is possible for the treatment center to negotiate a lower rate with local facilities to defray some of the expense. Residence Inn (www.marriott.com/residence-inn) is one such hotel that offers amenities to meet the unique needs of guests undergoing medical treatment, including simple services such as hotel staff stocking the refrigerator with unique patient food/drink needs. American Cancer Society's guest room program may provide short term lodging to patients, caregivers, or advocates. This lodging can be free or at a special medical rate at local hotels, depending on availability. Arrangements must be made through the local American Cancer Society office. Texas Medicaid recipients may be eligible for lodging at participating hotels, when they are traveling more that 100 miles for medical treatment.

Other resources used to identify services for patients and their families include but are not limited to chaplains, social workers, and home health agencies. Social services providing food include local churches and local food banks. Elderly patients may qualify for home delivery of food. Meals on Wheels is a program that deliver meals to individuals at home or hotel room who are unable to purchase or prepare their own meals. Because they are housebound, many of the recipients are elderly, and many of the volunteers are also elderly but able to drive and serve. The program guarantees that the recipient will have contact with the volunteer who delivers one hot meal per day.

Patients may apply for financial assistance in a number of ways. Some providers establish their own application process. Other providers may have specific guidelines which allow them to offer deep discounts or even write off entire balances to patients who qualify. Many foundations exist for the purpose of assisting with the costs of cancer care. It is essential that the patient or family member provide required documentation of financial need. The application can be stressful and confusing for a patient who is facing treatment decisions. A knowledgeable patient advocate can assist with the process and help to ease the burden. Patient Navigation programs can incorporate the financial aspect of care and provide the interface and guidance to serve this critical patient need. Models that can be developed to optimize support of the Patient Navigation program should involve social services interaction, financial advisor support, family counseling and an established policy with the research program business

department to be interactive or provide assistance to the various outside financial assistance support which may be unique to individual patients.

Thus the proof of principle provided in this assessment largely involving Texas related assistance will likely be an opportunity that can be established throughout the USA. Providing assistance with the cost of cancer care requires a team approach. Programs addressing assistance with pre-certification, co-pays, non-experimental drugs, and imaging are critical. When an individual's needs are identified, financial counselors, coordinators, doctors and nurse navigators need to utilize every resource available to meet those needs. This is especially true when participating in phase I trials, which involve significantly more time and commitment by the patient and family.

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Cerebral Venous Thrombosis Secondary to Severe Iron Deficiency Anemia: A Case Study

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Summary

Cerebral venous thrombosis (CVT) has been associated with numerous etiologies and a myriad of symptoms. Although CVT in association with iron deficiency anemia (IDA) has been observed primarily in pediatric patients, very few cases have been reported in adults. Herein, we describe an encounter with a 28 year-old female who presented solely with a new onset headache due to transverse sinus thrombosis. Thrombophilia work-up was normal. She had no identifiable acquired causes of thrombosis. The patient demonstrated severe iron deficiency anemia secondary to myoma uteri causing menorrhagia.

Keywords: Cerebral Venous Thrombosis (CVT), Iron Deficiency Anemia (IDA), stroke, myoma uteri

1. Introduction

CVT is an uncommon type of stroke, which may account up to 0.5 to 1% of all strokes (Saposnik et al., 2011). Though definitive epidemiological studies on CVT are lacking, previous data suggests that it is rare (Saposnik et al., 2011; Ferro et al., 2001; Saadatnia et al., 2004; deVeber et al., 2001; Lanska & Kryscio., 2006; Kalbag & Woolf, 1967). Although CVT has been reported in adults, it has more commonly been observed in neonates and children in hospital-based studies (Ferro et al., 2004; Lancon et al., 1999). Furthermore, the ratio of adult females to males is 3:1 (Ferro et al., 2004; Coutinho et al., 2009).

CVT has a variable clinical presentation, requiring a high level of suspicion for diagnosis and management (Bousser et al., 1985; Masuhr et al., 2004; Saposnik et al., 2011). It has been found that two mechanisms may principally lead to the clinical features of CVT. The first is the thrombosis of cerebral veins or dural sinus leading to cerebral parenchymal lesions or dysfunction. The second being the occlusion of the dural sinus resulting in decreased cerebral spinal fluid (CSF) absorption and elevated intracranial pressure (ICP).

CVT has been classified into three syndromes based on signs and symptoms: (1) isolated intracranial hypertension syndrome (Biousse et al., 1999), (2) focal syndrome (focal neurological deficits and/or seizures), (3) encephalopathy (multifocal signs, mental status changes, stupor, and coma) (Ferro et al., 2001; Bousser & Russell, 1997). In addition, clinical symptoms of CVT may simulate neurological diseases like stroke, brain tumor, and encephalopathy (Huang et al., 2010). Although CVT may present with any of the aforementioned symptoms, the most frequent is headache (Saposnik et al., 2011).

Diagnostic imaging by MRI in combination with MRV is the single most sensitive technique for demonstrating CVT (Stam, 2005; Dormont et al., 1994; Lafitte, 1997; Connor & Jarosz, 2002; Liang et al., 2001; Wasay & Azeemuddin, 2005). Although MRA, 3D-CT, CT venography, and angiography are also alternative diagnostic studies (Volcy-Gómez et al., 2003; Saposnik et al., 2011). The need for invasive cerebral angiography is uncommon and reserved for inconclusive MRV and CTV results (Bousser, 2000; Lafitte et al., 1997; Yoshikawa et al., 2002). Although rare, deep CVT may also be challenging to diagnose due to its non-specific neuroradiological and clinical features (Huang et al., 2010). However, the most frequent location of CVT

includes the superior sagittal sinus (62%), followed by the lateral or transverse sinuses (41-45%) (Saposnik et al., 2011).

Overall, 80% of patients with CVT have a good prognosis (Ferro et al., 2004), especially when intracranial hypertension is the sole manifestation. In contrast, approximately 5% of patients die in the acute phase due to neurologic sequelae, most commonly brain herniation, whereas 10% of patients die secondary to long term sequelae. A poor prognosis is associated with deep CVT as well as altered mental status (Ferro et al., 2004; Azin & Ashjazadeh, 2008). The recurrence of CVT is relatively uncommon with rates of only 2-7% (Ferro et al., 2004; Gosk-Bierska et al., 2006). Treatment for CVT includes antithrombotic therapy as well as symptomatic treatments (Einhäupl et al., 2010; 2006).

CVT is multifactorial and has been associated with the following: inherited hypercoagulable state, myoma uteri, pregnancy, puerperium, cancer, head trauma, intracranial or systemic infections, vasculitis, inflammatory bowel disease, dehydration, oral contraceptives, substance abuse (Stam, 2003; Ferro, 2006), and Behcet's disease (Abdulkader et al., 1995). Review of the literature reveals very few cases of reported CVT secondary to IDA. We describe here in a case of transverse sinus thrombosis secondary to IDA in a young female patient as a consequence of myoma uteri and menorrhagia.

2. Case Report

A 28-year-old Asian female without significant comorbidity presented to our hospital with headache as the sole complaint for the past 2-3 weeks. The patient denied any history of previous migraines, chronic/recurrent headaches, recent head trauma, vomiting, or fever. However, a history of sporadic metro-menorrhagia with recent heavy menorrhagia during her last menstrual cycle was elucidated. The patient's hematologic work-up is illustrated in Table 1, it was consistent with iron deficiency anemia, additional laboratory values revealed serum iron of 6 µg/dL, TIBC of 572 µg/dL, transferrin of 400 mg/dL, and iron saturation of 1%. Celiac sprue studies were normal. Patient was subsequently transfused with 3 units of packed red blood cells and discharged home after hemoglobin returned to 11.2g/dL. The patient was referred to a gynecologist for further assessment and management. However, she returned to the hospital hours later due to a worsening headache.

She denied any neurological symptoms except severe headache, further neurological evaluation revealed no focal deficits. A CT scan revealed complete thrombosis of the left transverse sinus with associated parenchymal hemorrhage, either within the parietal lobe or the left posterior fossa, adjacent to the tentorium. MRI/MRV of the brain with and without contrast revealed confirmation of transverse sinus thrombosis (Figures 1a and 2a), with focal thrombosed tributary rather than intraparenchymal hemorrhage. After identifying the presence of thrombotic formation, etiological factors for thrombosis such as the use of oral contraceptives, family and personal history of thrombosis, pre-existing SLE and coagulation disorders such as factor V Leiden, prothrombin G20210A mutation, excessive factor VIII levels and PNH were ruled out. In addition, a coagulation panel was ordered revealing a PT of 12.0 seconds, and INR of 1.0, PTT of 25 seconds, and platelet count of 240,000 mm^3 with additional normal laboratory values for anti-cardiolipin antibodies, B-2 glycoprotein I antibodies, antithrombin panel (AT), lupus anticoagulant panel (LAC), protein C and S, and homocysteine levels. During this hospitalization, a gynecology consult and imaging studies revealed the presence of myoma uteri, which was then surgically managed at a later time. She received intravenous iron to match her calculated iron deficit.

The patient was subsequently anticoagulated with heparin drip followed by warfarin daily until a therapeutic INR of 2.5 was achieved. Upon discharge, the patient was instructed to continue warfarin and oral iron therapy. Follow up MRV performed 8 weeks later demonstrated recanalization of the left transverse sinus. In addition, iron studies revealed a resolution of the anemia as shown in Table 1.

Table 1. Comparison of hematological values before and after treatment

Test	Normal Values	Before treatment	After Treatment
Hemoglobin	12.1-15.1g/dL	6.2 g/dL	13.3 g/dL
MCV	80-95fL	55 fL	95.3 fL
Ferritin	13-150 ng/mL	3 ng/mL*	135 ng/mL
Platelet count (Thousand/µL)	150-400	240	185

MCV=Mean Corpuscular Volume.
*Serum Ferritin is the most powerful test for the diagnosis of Iron Deficiency Anemia (Guyatt et al., 1992)

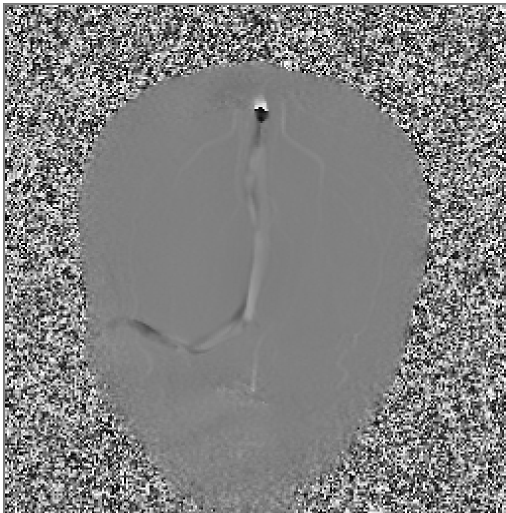

Figure 1a. MRV showing Left transverse sinus thrombosis

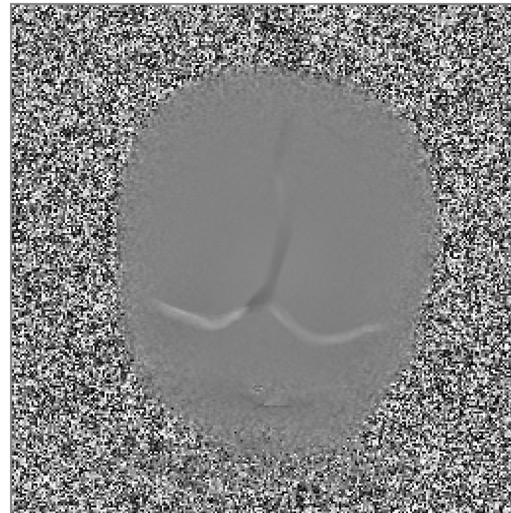

Figure 1b. MRV showing recanalization of left transverse sinus thrombosis after treatment

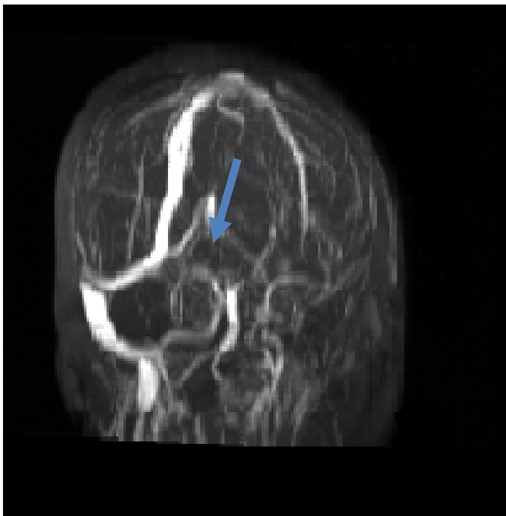

Figure 2a. MRV showing left transverse sinus thrombosis

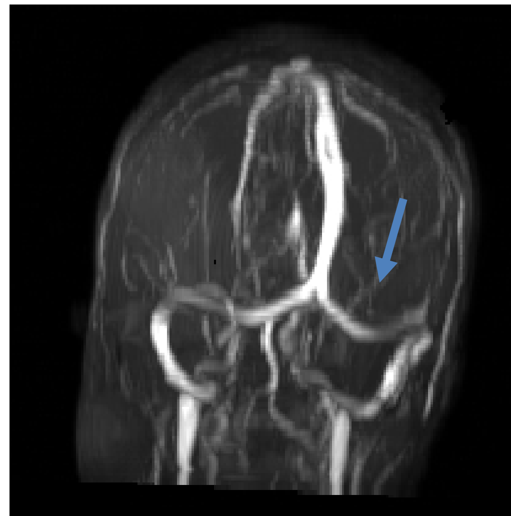

Figure 2b. MRV showing recanalization of left transverse sinus thrombosis after treatment

3. Discussion

CVT is an uncommon cause of stroke, which affects approximately five people per million per annum (Saposnik et al., 2011). As it is multifactorial, extensive investigations are often essential once the diagnosis is established. Around 85% of patients with sinus thrombosis may either have a prothrombotic risk factor or have an identifiable direct cause (Stam, 2005). As mentioned earlier, CVT can be associated with various risk factors, but CVT in association with IDA is extremely rare.

IDA is a worldwide problem and is relatively more frequent in children than in adults (Volcy - Gómez et al., 2003). Iron deficiency has been considered to be largely responsible for anemia, as circulating red blood cells have largest quantity of iron in the body (Cook et al., 1992). However, because of the role of iron in multiple processes at the cellular level (Yager & Hartfield, 2002), its deficiency can affect almost all organ systems including the brain. Neurological symptoms may include irritability, headaches, developmental delays (Yager, Hartfield, 2002; Hartfield et al., 1997) and, uncommonly, papilledema (Forster, 1985; Biousse et al., 2003), pseudo tumor cerebri (Parag & Omar, 1983), cranial nerve abnormalities like VI nerve palsy (Bruggers et al., 1990) and memory disturbances (Anezaki et al., 1992). Iron deficiency is seldom documented as an important trigger for stroke in chidren or adults (Hartfield et al., 1997).

To illustrate the relationship between IDA and CVT, the following mechanisms have been proposed:

(1) Thrombopoiesis is significantly regulated by iron (Karpatkin et al., 1974; Beguin, 1999), as normal quantity of iron is fundamental not only to maintain platelet production but also to prevent thrombocytosis. Thus, iron deficiency occasionally leads to thrombocytosis, which is associated with a hypercoagulable state. However, few cases of thrombocytopenia have been reported (Gupta & Joseph, 2001). According to Karpatkin et al., when iron deprivation occurs, it first leads to thrombocytosis; once the iron deficiency is severe enough to deplete iron, thrombocytopenia occurs (Karpatkin et al., 1974).

(2) Iron deficiency may also induce a hypercoagulable state by altering pattern of blood flow within the vessels due to decreased deformability and increased viscosity i.e. thickness of microcytic RBC (Hartfield et al., 1997).

(3) Low hemoglobin causes poor oxygenation. As a result, anemic hypoxia consequent to IDA could precipitate situations of increased metabolic stress predominantly in susceptible areas of the brain like basal ganglia and thalamus, due to end arterial blood supply (Balci et al., 2007). This fact could elucidate association of reversible focal deficits and stroke with IDA noticed by some authors (Hartfield et al., 1997; Young et al., 1983; Hart & Kanter, 1990).

Hypercoagulability, hemodynamic changes (either stasis or turbulence), and endothelial injury play important role in the thrombosis formation, according to Virchow's triad. Among these, hypercoagulability and stagnant flow predominate in thrombus formation in IDA (Ho et al., 2008). Although anemia causes increased arterial blood flow velocity (Aliefendioglu et al., 2007; Akins et al., 1996), it contributes to stasis in veins as a result of reduced deformability of microcytic RBC, which further leads to increased viscosity (Hartfield et al., 1997; Franchini et al., 2008). Intravascular thrombogenesis also caused by acute bleeding, as it augments platelet adhesiveness and reduces fibrinolytic activity (Ogata et al., 2008).

Although IDA commonly causes thrombocytosis, in our patient the number of platelets was not increased. To explain this association we have taken two studies into account:

(1) One study on iron deficiency and thrombosis reported that thrombocytosis is only a contributing factor for thrombosis as one third of cases had relatively normal platelet counts (Keung & Owen, 2004).

(2) Another study is a case control design by Stolz et al., in which data of a whole blood count and screening for thrombophilic coagulation abnormalities of 121 prospectively identified patients with CVT and 120 healthy controls were compared. In this study, severe anemia defined as hemoglobin <9 g/dl was independently and significantly associated with CVT of non-infectious origin, which might be interpreted as a higher dependence of hypercoagulability on the hemoglobin and hematocrit levels rather than on the extent of thrombocytosis. Despite the fact that this report did not specify the type of anemia and did not include the systemic analysis of iron metabolism, severe anemia was microcytic in 63% of cases with a female predominance. Hence, in most cases, iron deficiency anemia can be assumed (Stolz et al., 2007).

The association of IDA with sinus thrombosis has been reported previously in children (Sébire et al., 2005). However, only a few cases of adults with IDA have been reported (Balci et al., 2007; Ho et al., 2008; Ogata et al., 2008; Kinoshita et al., 2006; Aoki & Sakai, 1989). However, some of these cases were accompanied by other recognized risk factors of CVT, such as dehydration (Kinoshita et al., 2006), a hypercoagulable state (acquired protein C and protein S deficiency) (Ho et al., 2008), and cryoglobulinemia (Ho et al., 2008). These factors may have synergistic effects in addition to IDA and contributed to the CVT.

IDA is not a disease itself, but a manifestation of an underlying disease; Searching for the latter is therefore crucial and may be of far greater importance to the ultimate well being of the patient than just repleting iron stores (Rüfer et al., 2006). While the main cause of IDA in children is inadequate dietary intake or absorption, blood loss is the most common cause in adults. Blood loss could be due to hemoptysis, urine loss (hemoglobinuria, hemosiderinuria), gastrointestinal loss (parasites, ulcer, and malignancy), and from excessive menstruation. While the most common cause in adult men and post-menopausal women is GI blood loss, the commonest cause in pre- menopausal women is menstrual blood loss (Goddard et al., 2000). So far, two case reports have been published, wherein women with IDA presented with myoma uteri as a likely cause for CVT. One study reported hemorrhagic infarction in two women as a result of superior sagittal and transverse sinus thrombosis (Aoki & Sakai, 1989). Another study reported a woman with superior sagittal sinus thrombosis (Huang et al., 2010). These two reports as well as ours suggest that severe IDA secondary to myoma uteri related menorrhagia played a major role in the occurrence of CVT.

Although management of patients with CVT must be individualized, the basic therapy continues to be anticoagulation, which is intended to prevent propagation of thrombus and to increase recanalization. In all cases,

initial treatment consists of adjusted IV heparin dose or weight-based LMWH in full anticoagulant doses, followed by vitamin K antagonists, even with intracranial hemorrhage (ICH) (Einhäupl et al., 2010; 2006). Anticoagulation is not contraindicated for patients with intracranial hemorrhage that is resulting from CVT (Saposnik et al., 2011). In patients with transient risk factors, vitamin K antagonists may be continued for 3 to 6 months, to achieve the target INR of 2.0-3.0. Endovascular therapy i.e. thrombolyis or thrombectomy may be considered if anticoagulation is absolutely contraindicated or in case of initial treatment failure (Bousser, 2000; Masuhr, 2004; Stam, 2005). Steroids are not suggested, even with parenchymal brain lesions on CT/MRI if not warranted by another underlying disease (Canhão et al., 2008). For all patients therapy should be given for prevention of complications and symptomatic therapy should be given for seizures and ICP if present. Our patient received heparin and oral warfarin. The current initial therapy for patients with IDA is oral iron supplementation, which is inexpensive, nontoxic, and effective at correcting IDA. However, some patients do not tolerate it well, and in a subset of patients, it is insufficient, in which case parenteral supplementation is necessary (Wimbley & Graham, 2011). In this case, the patient was initially treated with intravenous iron followed by oral iron sulfate 325 mg TID, and was monitored with serial CBC and serum ferritin levels. Follow up MRI in our patient showed sinus recanalization (Figures 1b and 2b). However, in adults, limited data suggests that recanalization of the occluded sinus is not related to outcome after CVT (Strupp et al., 2002; Raizer & DeAngelis, 2000).

To summarize, this case suggests a connection between CVT and severe IDA, in this setting, iron deficiency should be considered as an underlying cause of CVT in not only pediatric population but also in adults. Despite the fact that in women of childbearing age, CVT can occur due to oral contraceptives, pregnancy and puerperium, special attention should be given to middle-aged women, as IDA is a predisposing disorder to sinus venous thrombosis. It is always important to look for menorrhagia, as they underestimate the extent of menstrual losses (McKenna et al., 1989). Recurrence of CVT could be significantly prevented by supplementation therapy for iron deficiency. Comprehensive treatment for IDA is required, as in the acute phase of CVT, anemia is frequently noticed as a relatively low hemoglobin (Hb) concentration (Sébire et al., 2005) and particularly in patients with other significant thrombotic risk factors should be treated actively.

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The Anti-Cancer Effect of a Novel Nutrient Mixture by Inhibiting MMPs Expression, Invasion and Inducing Apoptosis in Chondrosarcoma Cell Line SW-1353

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Abstract

Chondrosarcoma, a malignant tumor of cartilaginous origin is the most frequent adult primary bone cancers. Surgery is the main treatment option because chondrosarcoma typically does not respond to radiation and chemotherapy. Cancer mortality usually results from the local and distant metastasis. A nutrient mixture containing ascorbic acid, lysine, proline, green tea extract, was tested on chondrosarcoma cells SW-1353 for viability, matrix metalloproteinase expression, Matrigel invasion, morphology and apoptosis. The SW-1353 cells were grown in appropriate media and treated with the nutrient mixture at 10, 50, 100, 500 and 1000 μg/ml concentrations. Cells were also treated with PMA (100 ng/ml) for MMP-9 stimulation. Cell proliferation was carried out by MTT assay, MMPs by zymography, invasion through Matrigel. Morphology and apoptosis were also conducted. The nutrient mixture did not exhibit toxicity at 100 μg/ml, but showed 40% inhibition at 1000 μg/ml. Zymography demonstrated two bands, for MMP-2 and MMP-9. PMA treatment further enhanced MMP-9 expression. The nutrient mixture inhibited expression of both MMPs in a dose dependent manner. Matrigel invasion was reduced by 40%, 70%, 88% and 100% at 50, 100, 500 and 1000 μg/ml respectively. The nutrient mixture induced slight apoptosis at 250 μg/ml, moderate at 500 μg/ml and significant at 1000 μg/ml concentrations. Morphology showed slight changes at the highest concentrations. The nutrient mixture significantly inhibited all the important hallmarks for cancer progression, suggesting that it has a possibility for exploration as a significant therapeutic entity in chondrosarcoma.

Keywords: chondrosarcoma, nutrient mixture, MMP, Matrigel invasion, apoptosis

1. Introduction

Chondrosarcoma, a malignant tumor of cartilage cells mainly affecting adults between 30-60 years. According to the American Cancer Society 2012 estimates, chondrosarcoma has become the most common primary bone cancer accounting for more than 40% of the adult primary bone tumor cases. It grows in the chondrocytes (cartilage cells), and mainly affects the legs, upper arms, scapula, ribs and pelvic bones. Although the exact cause of chondrosarcoma is not known, it is postulated that individuals with certain genetic or chromosomal abnormalities are at increased risk. Grade and stage are the most important and independent prognostic factors for survival in chondrosarcoma. Cancer mortality usually occurs from tumor invasion resulting in local or distant metastasis to vital organs. High-grade chondrosarcoma has much poorer prognosis. 10-year survival of high-grade chondrosarcoma was calculated to be 38% (Fiorenza et al., 2002). Recurrence is based on irregular tumor margins and lesions greater than 10 cm. Surgery is generally the main treatment option for chondrosarcoma. Chemotherapy or radiation therapies are not very effective and are rarely given in cases of extensive metastasis (Gelderblom et al., 2008). The high metastatic potential and inadequate treatment methods leading to poor outcomes, require an urgent need to develop more effective less toxic treatment alternatives.

We have developed strategies to inhibit cancer growth and expansion using naturally occurring nutrients including lysine, proline, ascorbic acid, green tea extract, and others. According to Rath and Pauling research (1992), nutrients such as lysine and ascorbic acid have been suggested to act as natural inhibitors of extra cellular matrix (ECM) degradation, and as such have potential to modulate tumor growth and expansion. These nutrients can exert their effects by strengthening the connective tissue surrounding cancer cells by increasing collagen

synthesis, as well as inhibit the expression of matrix metalloproteinase (MMP) enzymes. In our previous studies, these nutrients (the nutrient mixture, NM) have exhibited broad spectrum therapeutic and chemoprotective activities in vitro and in vivo in a number of cancer cell lines. This synergistic anticancer effects of the NM was observed by inhibition of cancer cells growth, expression of MMPs, Matrigel invasion, metastasis, and angiogenesis (Roomi et al., 2006, 2007, 2009a, 2009b). Considering the efficacy of NM on other cancer cell lines, we investigated the effects of NM on the human chondrosarcoma cell line SW-1353. We hypothesized that NM would significantly inhibit the growth, MMP expression and invasion of the SW-1353 cancer cell line.

2. Materials and Methods

2.1 Composition of Nutrient Mixture

The composition of the nutrient mixture (NM) and the proportion included the following: Vitamin C (as ascorbate salts of Mg, Ca and plamitate) 710 mg; L-lysine 1000 mg; L-Proline 750 mg; L-Arginine 500 mg; N-Acetyl Cysteine 200mg; Standardized Green Tea Extract (80% polyphenol) 1000 mg; Selenium 30 µg; Copper 2 mg; and Manganese 1 mg.

2.2 Cancer Cells and Culture

Chondrosarcoma cells line, SW-1353 was from American Type Culture Collection (ATCC, Rockville, MD), and grown in modified Dulbecco's Eagle medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco BRL, Long Island, NY) in 24-well tissue culture plates (Costar, Cambridge, MA). Cells were incubated in 1 ml of media at 37°C in a tissue culture incubator equilibrated with 95% air and 5% CO2. At near confluence, the cells were treated with the NM, dissolved in the media and tested in triplicate: 0, 10, 50, 100, 500 and 1000 µg/ml. Cells were also treated with Phorbol Myristate Acetate (PMA) 100 ng/ml to enhance MMP-9 expression. The plates were then returned to the incubator.

2.3 MTT Assay

Cell viability was carried out by MTT assay. The colorimetric assay based on the ability of viable cells to reduce a soluble yellow tetrazolium salt [3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazilium bromide] (MTT) to a blue formazan crystal by mitochondrial succinate dehydrogenase activity of viable cells. This method is a good index of mitochondrial activity and thus of cell viability. After a 24 hour incubation, the cells were washed with phosphate buffered saline (PBS) and 500 µl of MTT (Sigma#M-2128) 0.5mg/ml in media was added. After addition the plates were returned to the 37°C incubator for 2 hours. After which, the supernatant was removed, 1 ml of DMSO was added, and absorbance was read at 570 nm in Bio Spec 1601, Shimadzu spectrometer. The OD570 of the DMSO solution in each well was considered to be proportional to the number of cells. The OD of the control (treatment without supplement) was considered 100%.

2.4 Gelatinase Zymography Enzyme Activity Assay

Gelatinase zymography was carried out in 10% Novex Pre-Case SDS Polyacrylamide Gel (Invitrogen Corporation) in the presence of 0.1% gelatin under non-reducing conditions. Culture media (20 ml) were mixed with sample buffer and loaded for SDS Plyacrylamide Gel Electrophoresis (SDS-PAGE) was performed as suggested by the manufacturer (Novex). Samples were not heated before electrophoresis. Following electrophoresis the gels were washed twice in 2.5% Triton X-100 for 30 minutes to remove SDS. The gels were then incubated at 37°C overnight in $CaCl_2$ at pH 8.0 and stained with 0.5% Coomassie Blue R250 dissolved in 50% methanol and 10% glacial acetic acid and destained. Upon renaturation of the enzyme, the gelatinases digested the gelatin in the gel, producing clear bands against an intensely stained background. Protein standards were also ran and approximate molecular weights werecalculated. Gelatinase zymograms were scanned using CanoScan 9950F Canon scanner at 1,200 dpi. The intensity of the bands was evaluated using a pixel-based densitometer program Un-Scan-It, Version 5.1, 32-bit, (Silk Scientific Corporation) at a resolution of 1 Scanner Unit (1/100 dpi), and expressed as a percentage of control.

2.5 Matrigel Invasion Assay

Invasion studies were conducted using Matrigel (Becton Dickinson) inserts in 24-well plates. Suspended in medium, SW-1353 cells were supplemented with the NM and seeded into the inserts in the well. Thus, both the medium on the insert and in the well had the same supplements. The plates were then returned to the incubator and equilibrated with 95% room air and 5% CO_2. After incubation, the media from the wells were drawn. The cells on the upper surface of the inserts were gently scrubbed away with cotton swabs. The cells that had penetrated the Matrigel membrane and migrated onto the lower surface of the Matrigel were stained with hematoxylin and eosin (H & E) and counted under the microscope.

2.6 Morphology and Apoptosis

Morphology of the cells cultured in test concentrations of were evaluated after 24 hours by H&E staining and apoptosis using Live Green Caspase Detection Kit and photographed by microscope. The SW-1353 cells were challenged with NM dissolved in media at the experimental doses and incubated for 24 hours. The cell culture was washed with PBS and treated with the caspase reagent as suggested by the manufacturer (Molecular Probes Image-IT Live Green Poly Caspases Detection Kit 135104, Invitrogen). The photographs were taken with a fluorescence microscope and cells were counted. Green-colored cells represent viable cells, while yellow orange and red represents early and late apoptosis respectively.

2.7 Data Analysis

The results were expressed as mean ± standard deviation. Data was analyzed by independent t-test. Significance was determined at $p<0.05$.

3. Results

3.1 Cell Proliferation

Figure 1 shows the effects of NM relative to control in triplicate of the Chondrosarcoma cell line. NM was not toxic to chondrosarcoma cell line SW-1353 at 100 μg/ml, but exhibited 10% and 40% ($p<0.001$) toxicity at both 500 and 1000 μg/ml, respectively.

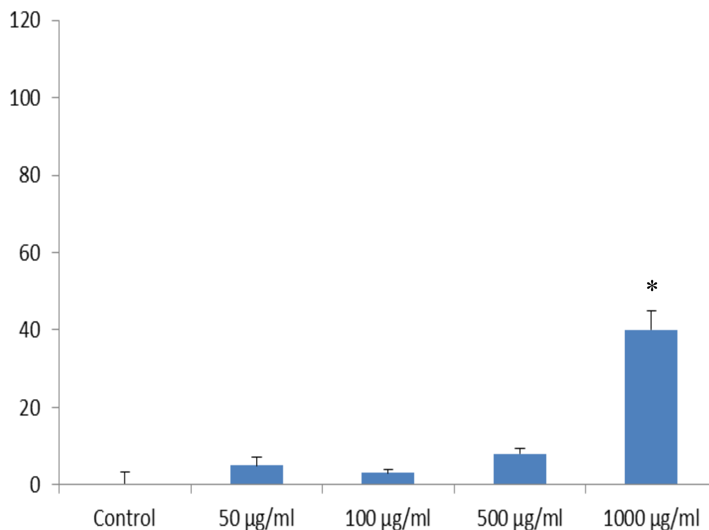


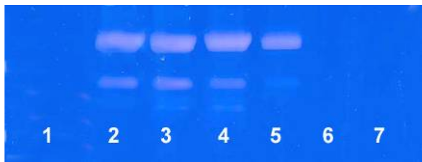
Figure 1. Effect of the NM on chondrosarcoma cell line SW-1353 proliferation

The cell proliferation was inhibited 10% at 500 μg/ml and 40% at 1000 μg/ml. (* Significant at $p<0.001$)

3.2 Gelatinase Zymography

Zymography demonstrated two bands corresponding to MMP-2 and MMP-9. PMA (Phorbol Myristate Acetate) treatment stimulated MMP-9 expression. NM inhibited the expression of both MMP-2 and MMP-9 in a dose-dependent fashion; 50% at 100 μg/ml and 100% at 500 μg/ml (Figure 2A and 2B). Densitometry analysis of MMP-2 for untreated SW-1353 cells showed inhibition of 56% at 100 μg/ml and 98% at 500 μg/ml of NM. Regression analysis showed a linear trend of R2=0.85 (Figure 2C). For SW-1353 cells treated with PMA, MMP-2 inhibition was seen as follows: 97% at 50 μg/ml, 98% at 100 μg/ml and 100% at 500 μg/ml. Regression was noted at R2=0.81 (Figure 2D). Untreated SW-1353 cells showed an inhibition of MMP-9 expression of 33% at 10 μg/ml, 98% at 50 μg/ml, 99% at 100 μg/ml and 100% at 500 μg/ml. R2 for these cells was 0.88 (Figure 2C). Finally, for the SW-1353 cells treated with PMA, an equally strong inhibition of expression of MMP-9 was observed. MMP-9 expression was inhibited by 82% at 50 μg/ml of NM, 83% at 100 μg/ml and 99% at 500 μg/ml. Regression coefficient for PMA-induced MMP-9 expression of SW-1353 cells was calculated to be R2=0.86 (Figure 2D).

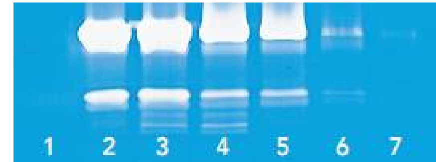
2A- Normal SW-1353 cells



MMP-9

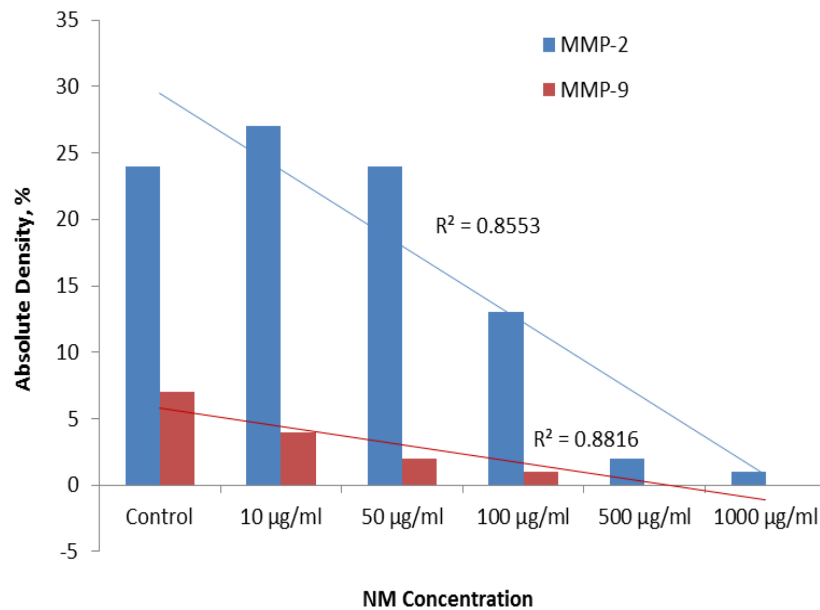
MMP-2

2B- SW-1353 cells treated with PMA



The upper row corresponds to MMP-9 secretion and the lower line to MMP-2. Each column corresponds to increasing concentration of NM, (Lane 1:Marker, 2:Control, Lanes 3-7: NM doses 10, 50, 100, 500 and 1,000 µg/ml)

2C. Densitometry of MMP-2 and MMP-9 of SW-1353 cells. R^2 represents a linear regression analysis of the dose-dependent inhibition of MMP



2D. Densitometry analysis of MMP-2 and MMP-9 of PMA (100 ng/ml) treated SW-1353 cells

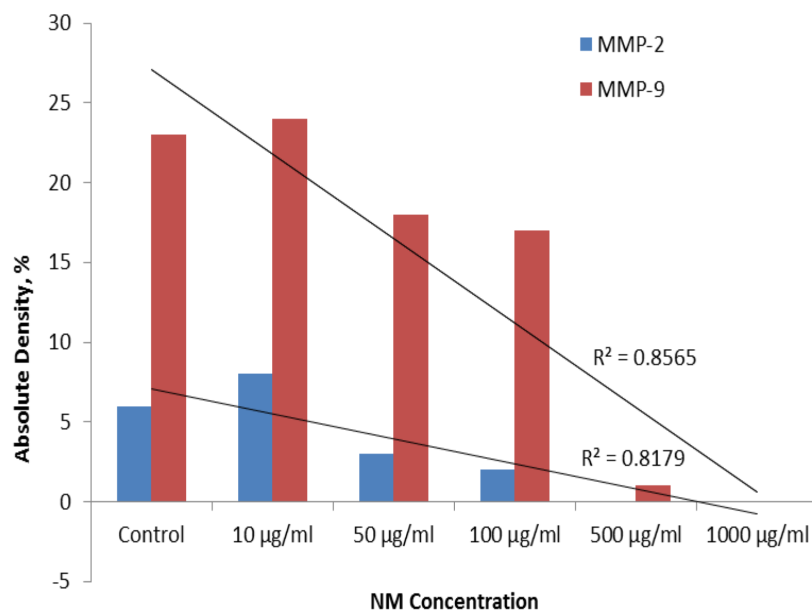
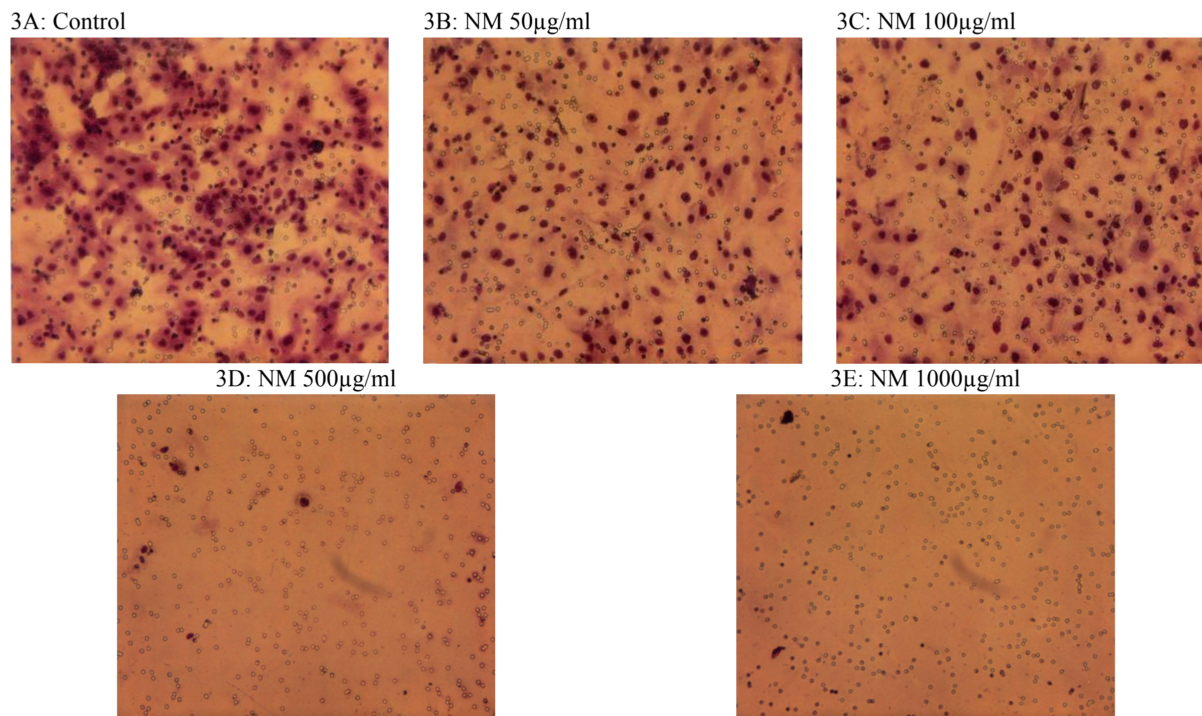


Figure 2. Effect of NM on MMP-2 and MMP-9 secretion by chondrosarcoma cell line SW 1353

3.3 Matrigel Invasion

Figure 3 (A-E) reveals a significant dose-dependent inhibition of SW-1353 cell migration and invasion through Matrigel membrane. 38% inhibition was observed at 50 µg/ml, 65% at 100 µg/ml, 85% at 500 µg/ml and 100% inhibition was noted at 1000 µg/ml of NM concentration, $p<0.001$ (Figure 3F).



3F: Effect of NM Matrigel invasion on SW-1353 cells (* Significance $p<0.001$ with respect to control)

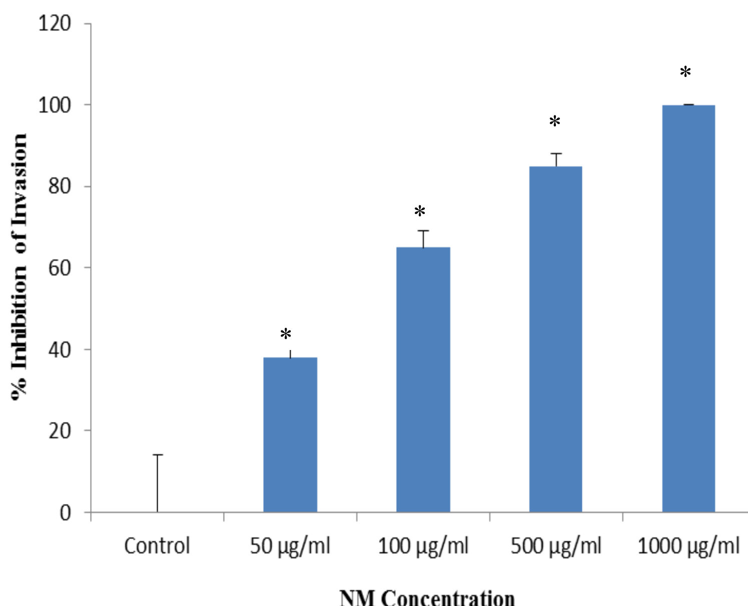


Figure 3. Effect of NM on chondrosarcoma cell line SW-1353 invasion through Matrigel (Magnification 10x)

3.4 Morphology and Apoptosis

H&E staining of SW-1353 cells exposed to NM showed no changes at the 50 µg/ml and 100 µg/ml doses but showed slight changes at the 500 µg/ml dose (Figure 4 A-E). These changes include, shrinking of the cytoplasm,

darkly stained nuclei and intensely acidophilic cytoplasm suggest cells undergoing apoptosis. Using the live green caspase kit, a dose-dependent apoptosis of the SW-1353 cells was evident with the NM. Figure 5 (A-E) highlights that as the concentration of NM was increased the apoptotic events also increased. A quantitative analysis of this is represented in Figure 5F, which shows concentration of live cells gradually decreased as the NM dose increased. At 250 µg/ml of NM, 81% cells were live, however at 500 µg/ml only 37% cells were live and 62% were either in early or late apoptosis. At 1000 µg/ml, 10% cells were live, while 90% cells were in early and late apoptosis stage.

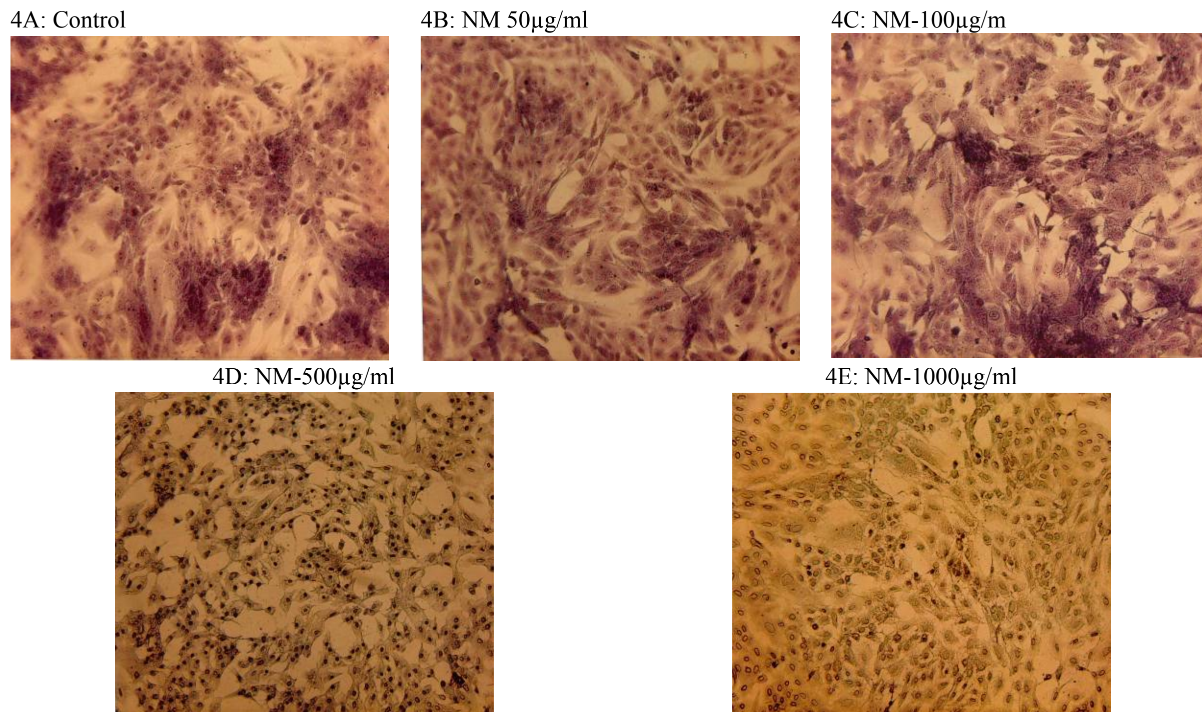


Figure 4. SW 1353 cell morphology showed slight changes at the higher concentrations

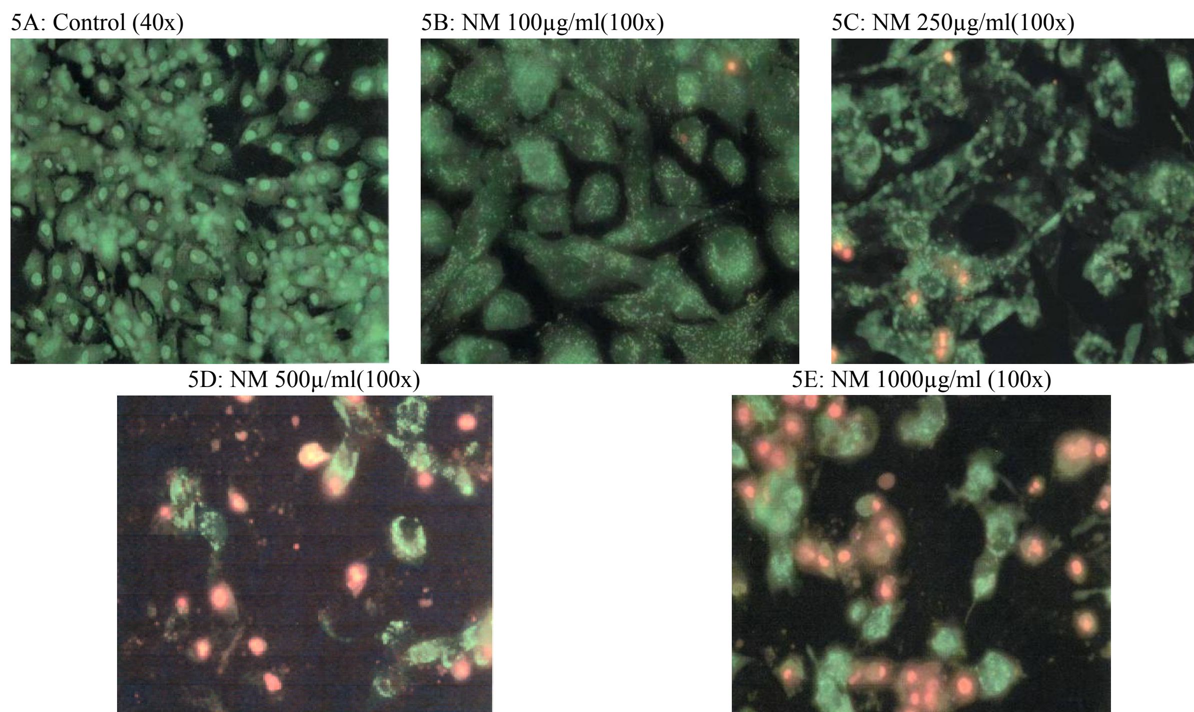


Figure 5F: Quantitative analysis of apoptotic events in SW-1353 cells exposed to NM

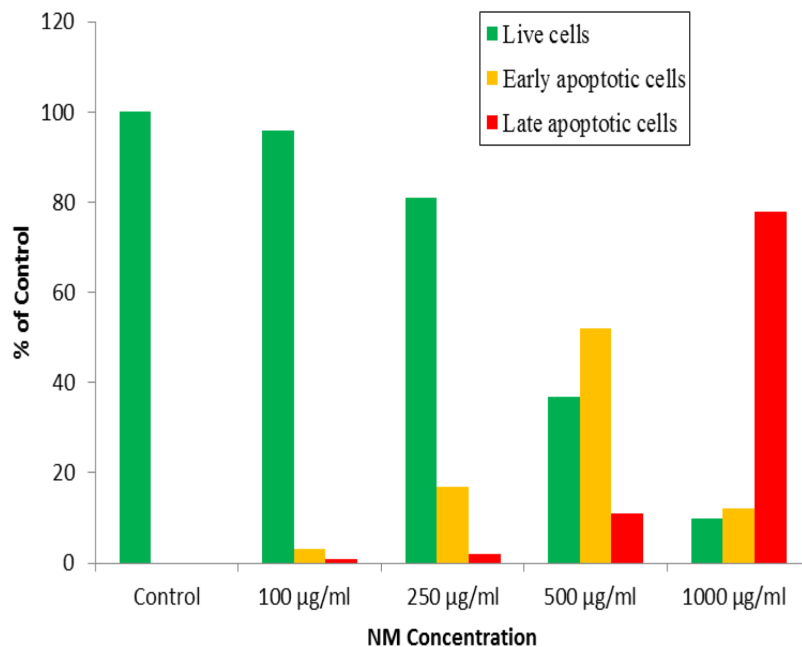


Figure 5. Photomicrograph of apoptotic events using live green caspase at increasing concentrations of NM

4. Discussion

The results of our experiments on the chondrosarcoma cell line SW-1353 suggest that NM is effective in inhibiting cell proliferation above a concentration of 500 µg/ml. Additionally, a dose-dependent decrease in MMP expression and Matrigel migration was observed. Furthermore, NM also induced apoptosis. These are the most important steps in cancer invasion and metastasis.

The NM formulation was based upon several important nutrients that exhibited anti-cancer properties, in particular those that reinforce the ECM, inhibit MMP activity and are cytotoxic to cancer cells. For example, ascorbic acid and the amino acids lysine and proline are critical to proper ECM formation and structural integrity by ensuring synthesis and hydroxylation of collagen fibers (Roomi et al., 2005). Lysine possesses an additional role in maintaining proper collagen formation by inhibiting proteolysis (Rath & Pauling, 1992). Green tea extract has shown promise in controlling cancer growth, metastasis and angiogenesis (Taniguchi et al., 1992; Valcic et al., 1996; Yang et al., 1998; Mukhtar & Ahmed, 2000). N-acetylcysteine is known to inhibit MMP-9 and invasive activities of tumor cells (Morini et al., 1999; Kawakami, Kageyama, Fujii, Kihara, & Oshima, 2001). In addition to regulating ECM properties, certain nutrients can induce cell death. Ascorbic acid, for example, is known to inhibit cell division and growth via hydrogen peroxide production (Maramag, Menon, Balaji, Reddy, & Laxmanan, 1997). Arginine, a known precursor of nitrogen oxide, also plays an important role in cell apoptosis induction (Cooke & Dzau, 1997). Finally, selenium has been shown to inhibit the secretion of MMP secretion and tumor invasion (Yoon, Kim, & Chung, 2001).

These nutrients can exert their effects by strengthening the connective tissue surrounding cancer cells by influencing collagen synthesis, as well as inhibit the expression of MMP enzymes. Free radical injury plays a key role in cancer initiation and progression. During the multistep process, the degradation of ECM by MMPs is a critical step in tumor growth, invasion, and metastasis. It is important to restrict this step to halt tumor progression. Matrix metalloproteinases are a family of proteolytic enzymes able to degrade connective tissue and are associated with cancer metastasis and tumor angiogenesis. Two key enzymes, MMP-2 and MMP-9, play a key role in the degradation of collagen types II and IV, important components of the ECM. Higher expression of MMP enzymes is shown to play a role in the malignancy potential of chondrosarcomas. When high-grade chondrosarcoma samples were compared to lower-grade samples using immunohistochemistry, the ratio (grade 3 to grade 1) of gene expression of MMP-2 exceeded (Boeuf, Bovée, Lehner, Hogendoorn, & Richter, 2010). In a comparison of MMP-2 and MMP-9 expression of normal cartilage and chondrosarcoma tissue, the chondrosarcoma cells samples had a higher expression of MMPs (Moses & Shing, 1994). Elliot et al. (2002)

showed that Bcl-3, inhibitor of NF-kappaB1 can cooperate to activate MMP-1 transcription of chondrosarcoma cells SW 1353. In another study, Elliot's group also showed that synthetic triterpenoid, CDDO, inhibited IL-1 induced MMP-1 and MMP-13 expression in SW-1353 cells (Elliott et al., 2003). While Burrage et al. evaluated the effect of synthetic ligand LG 100268 for nuclear hormone receptor and it selectively inhibit IL-1 and stimulate MMP-1 and MMP-13 (Burrage et al., 2007).

Our studies demonstrated significant inhibition of MMP-2 and MMP-9 expression, the important mediators of angiogenesis and metastasis. These data suggest NM may have a role in the therapeutic approaches of chondrosarcoma, specifically by targeting MMP expression and thereby inhibiting migration of chondrosarcoma within the ECM as well as stabilizing the ECM surrounding an encapsulated tumor, thereby reducing chances of metastasis. In addition, NM maximally inhibits the proliferation of cancer cells at high doses, inhibits Matrigel invasion and induces apoptotic changes at the cellular level. The effect appears to be cancer-specific since our previous studies demonstrated no NM toxicity on a variety of normal cells, such as fibroblasts, smooth muscle cells and endothelial cells (Ivanov, Ivanova, & Niedzwiecki, 1997; Ivanov et al., 2007). Furthermore, the NM has also been shown to be safe in vivo. In a previous in vivo study, we found that gavaging adult female ODS rats with the NM (at 3, 90 and 150 mg per day for seven days), had no adverse effects on vital organs such as the heart, liver, and kidneys. In the same study, the NM did not affect the associated serum enzymes indicating that this mixture is safe to use even at higher doses, by far exceeding the normal equivalent doses of this combination (Roomi et al., 2003). Overall, the NM may offer a therapeutic benefit and play a role in support of chondrosarcoma.

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Anticancer Properties of Hydroxycinnamic Acids -A Review

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The authors declare that they have no competing interests

Abstract

Hydroxycinnamic acid compounds are an important source of antioxidants due to their ubiquitous occurrence in the plant kingdom and their characteristic activities. Due to their antioxidant activity, several researchers have attributed a probable role of these compounds in the prevention of various diseases associated with oxidative stress, such as cancer and cardiovascular diseases. Recent evidence suggests that these compounds may also act by other mechanisms in addition to the antioxidant capacity as modulating the activity of some specific enzymes and inhibit cell proliferation. This paper is a comprehensive review of the effects of hydroxycinnamic acids on cancer. The review encompasses the occurrence and bioavailability of these compounds evidences for their effects on cancer and the various mechanisms by which may exert their effects. There are several common mechanisms by which these chemicals exert their effects that could be conducive to additive, synergistic, or antagonistic interactions. These include effects on cellular differentiation, proliferation, and apoptosis; effects on proteins and enzymes that are involved in these processes at a molecular level, and other various effects through altered immune function and chemical metabolism.

Keywords: hydroxycinnamic acids, chlorogenic acids, antioxidant, cancer

1. Introduction

Phenolic compounds are secondary metabolites of plants widely distributed in foods and beverages of plant origin. They are secondary metabolites of these plants and are involved in defense against ultraviolet radiation or aggression by pathogens (Manach et al., 2004). These compounds may be classified in phenolic acids and flavonoids (Farah & Donangelo, 2006).

Phenolic acids are molecules with one phenol ring bound with one or more hydroxyl groups, found in fruits, vegetables and products derivatives (Liu, 2004). These substances can be subdivided into two major groups: derivatives of hydroxybenzoic acids and derivatives of hydroxycinnamic acids (Figure 1) (Manach, Scalbert, Morand, Rémésy & Jiménez, 2004).

The hydroxybenzoic acids (Figure 1A) are components of complex structures of tannins and lignins and are less abundant in plants consumed by humans (Manach et al., 2004). Its derivatives include *p*-hydroxybenzoic, protocatechuic, vannilic, syringic and gallic acids. On the other hand, the hydroxycinnamic acids (Figure 1B) are the largest class of phenolic compounds (Huang, Johanning & O'Dell, 1986; Herrmann, 1989), represented by caffeic, *p*-coumaric and ferulic acids (Figure 1A) (Crozier, Jaganath & Clifford, 2009; Lafay & Gil-Izquierdo, 2008; Karakaya, 2004). Caffeic acid (CA) is the main hydroxycinnamic acid found in foods, mainly as chlorogenic acid (CGA).

Chlorogenic acids are formed by the esterification of (-)-quinic acid (Figure 2A) with one to four molecules of hydroxycinnamic acids (Clifford, 2000). The esters of this acid are formed preferably on carbon 5 of quinic acid (Figure 2B), but also on carbons 3 and 4, and less commonly on carbon 1 (Farah & Donangelo, 2006). The main subclasses of CGA are the caffeoylquinic acids (CQA), feruloylquinic acids (FQA) and dicaffeoylquinic acids

(diCQA), with at least 3 isomers per subclasses (Farah, Monteiro, Donangelo & Lafay, 2008; Monteiro, Farah, Perrone, Trugo, & Donangelo, 2007; Farah & Donangelo, 2006).

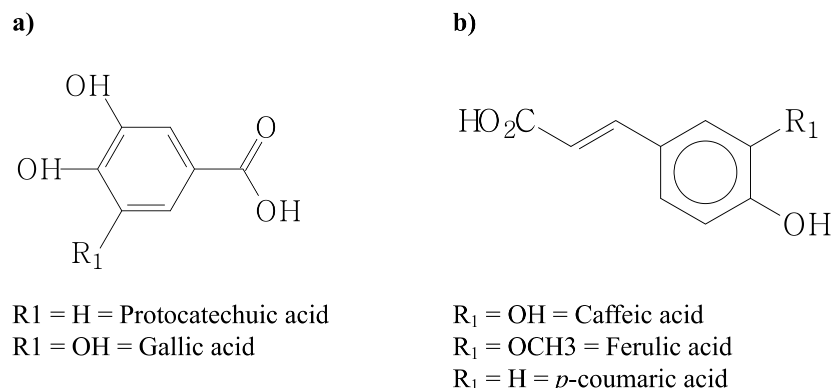

Figure 1. Chemical structures of hydroxybenzoic acids (A) and hydroxycinnamic acids (B)

CGA is found in many types of fruits (such as apples, blueberries, cherries, kiwis and plums), vegetables (chicory, potato and artichoke) and herbs (Ilex paraguariensis and Achyrocline satureioides), being coffee the main source in the Western diet (Marques & Farah, 2009; Manach et al., 2004; Clifford, 2000).

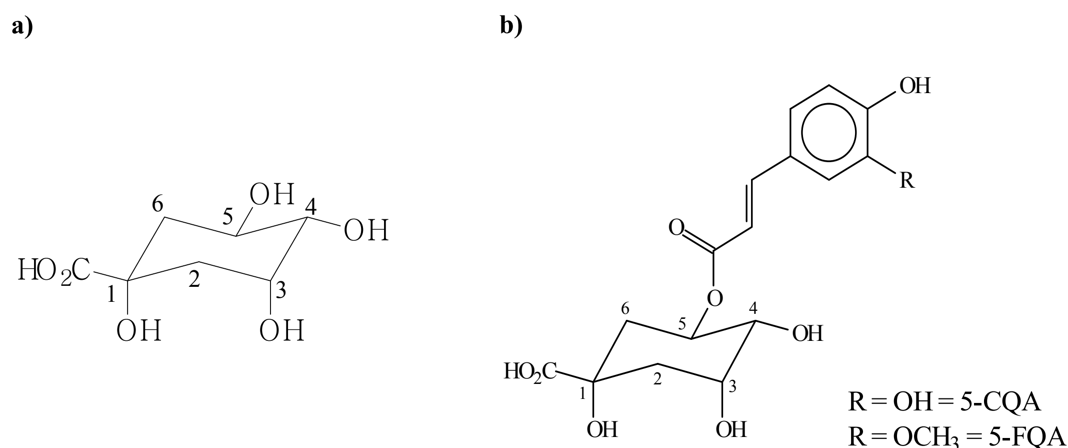

Figure 2. Chemical structures of (-)-quinic acid (A) and example of 5-isomers for CGA monoesters (B)

The presence of phenolic compounds in plants has been studied by participating in processes responsible for color, astringency and flavoring in different foods. Furthermore, due to their antioxidant activity, several researchers have attributed a probable role of these compounds in preventing of various diseases associated with oxidative stress, such as cancer and cardiovascular and neurodegenerative diseases (Manach et al., 2004; Johnston, Clifford, & Morgan, 2003; Natella, Nardini, Giannetti, Dattilo & Scaccini, 2002).

2. Metabolism, Absorption and Bioavailability of Hydroxycinnamic Acids

Until recently, only traces of CA and/or 5-CQA had been identified in both animal and human plasma (Lafay et al., 2006; Wittemer et al., 2005; Olthof, Hollman, Buijsman, Van Amelsvoort, & Katan, 2003; Rice-Evans, Spencer, Schroeter, & Rechner, 2000). However, currently it is known that the absorption of CGA is much higher than previously thought and studies have been shown that some individuals seem to be able to absorb up to 73% of the total of CGA ingested (Farah et al., 2008).

The maximum plasma concentration of total CGA in humans seems to be around 15 µmol/L and the predominant isomer of CGA, 5-CQA, has an average value of 6 µmol/L. Besides the main isomers of CGA, it is known that

some of its metabolites (caffeic acid, ferulic, isoferulic, and *p*-coumaric acid) are also bioavailable in humans with concentrations around 1.0 µmol/L (Farah et al., 2008; Monteiro et al., 2007). These values should be considered when carries out studies to evaluate the pharmacological properties of CGA, which often use concentrations a thousand times above these values.

Recently, Farah et al. (2008) and Monteiro et al. (2007) demonstrated for the first time that main isomers of CGA (3-, 4- and 5-CQA, 4- and 5-FQA, 3,4-, 3.5- and 4,5-diCQA) are bioavailable in the human body. These results indicated that a small percentage of CGA ingested are absorbed in stomach, been the major part absorbed and metabolized in the small intestine. Studies have shown that CGA follows intact toward intestine, where they would be conjugated, absorbed and/or metabolized (Duarte & Farah 2011; Marques & Farah, 2010; Monteiro, Marques & Farah, 2010). In rats, Choudhury et al. (1999) suggested that at least a small amount of 5-CQA ingested is absorbed preferentially by jejunum when compared to ileum (Figure 3).

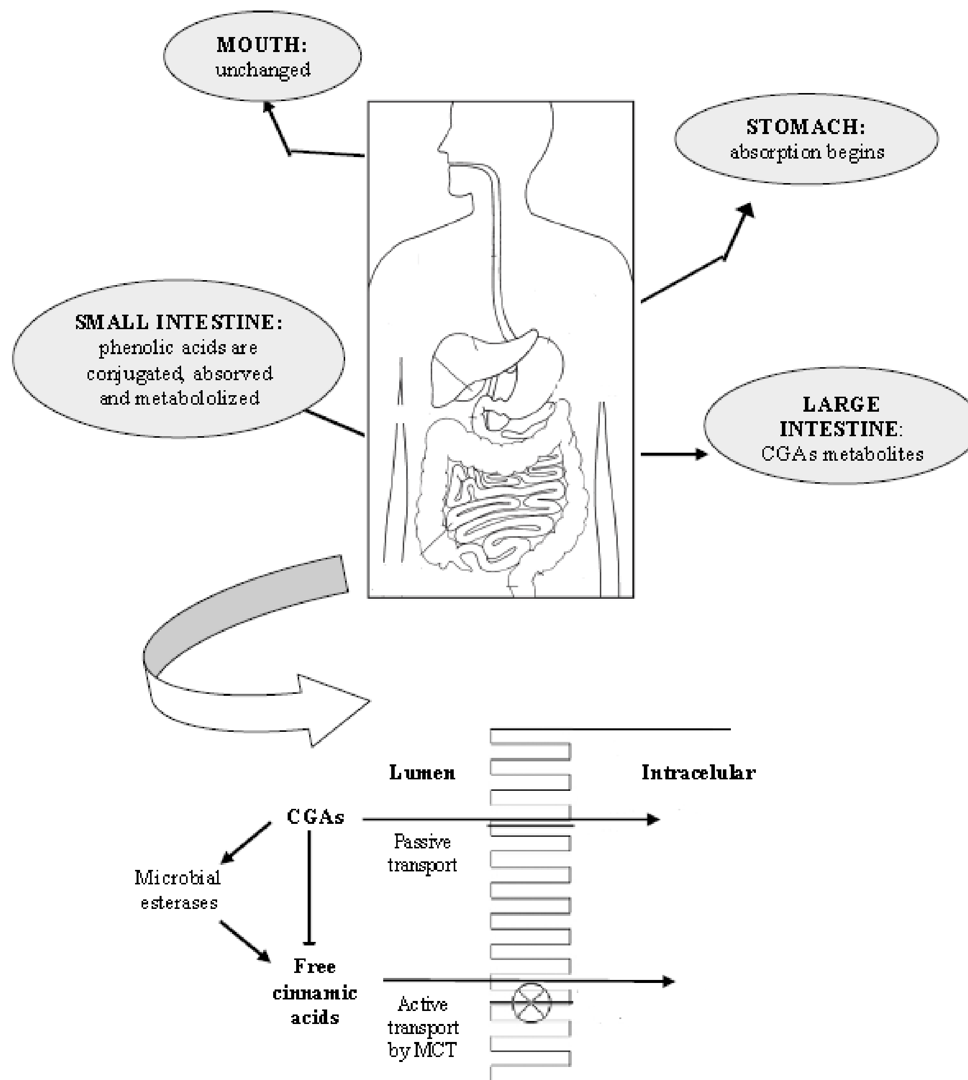

Figure 3. Chlorogenic and hydroxycinnamic acids absorption (Adapted from Bastos and Oliveira, 2011)

The CGA not absorbed by small intestine seem to follow until the large intestine, where they would suffer bacterial action. In fact, Couteau et al. (2001) reported that a large number of intestinal bacteria expressed esterase activity and are capable of hydrolyzing CGA to form caffeic acid and other metabolites in the intestine. These esterase activity are able to release cinnamic acid of CGA structure, which can then be absorbed and metabolized to its primary metabolites (caffeic acid, ferulic, isoferulic, and *p*-coumaric) (Couteau et al. 2001). Additionally, Konishi et al. (2004) demonstrated that CA liberated from CGA by intestinal mucosa esterase, is not only absorbed via paracellular diffusion but also actively absorbed by the monocarboxylic acid transporter

(MCT) (Figure 3).

Part of CGA appears to be directly absorbed by the portal system following to the liver, where they would then metabolized, stored and/or released gradually by bile for an enterohepatic circulation (Baer-Dubowska & Szaefer, 1998; Olthof et al., 2003; Manach et al., 2004). In fact, presence of CGA, CA, and ferulic and *p*-coumaric acids free in saliva, gastric and enteric fluids of human after 12 h of fasting has been reported in the literature (Monteiro & Farah, 2008). This would agree with the hypothesis proposed by Booth et al. (1957), in which the author suggested that 5-CQA could stand being stored in the human body.

3. Antioxidant Activity of Hydroxycinnamic Acids

Phenolic compounds have been widely studied due to their influence on food quality. They are constituted by a large amount of substances, among them hydroxycinnamic acids, which have antioxidant properties, as a result of their chemical structure.

Hydroxycinnamic acids have been consistently associated with reduced risk of cardiovascular disease, cancer and other chronic diseases (Spencer, El Mohsen, Minihane & Mathers, 2008). The ability of these substances in scavenges free radicals and pro-oxidant metals (antioxidant) partly explain this association. Recent evidence suggests that these compounds may also act by other mechanisms in addition to the antioxidant capacity as modulating the activity of some specific enzymes and inhibit cell proliferation (Manach, 2004). General antioxidant capability of the hydroxycinnamic acids *in vitro* can be expressed by the decreased malondialdehyde formation in several lipid peroxidation systems; scavenging of O_2 and decreased rates of OH' formation (Laranjinha et al., 1994).

Comparing the reducing power of hydroxycinnamic acids, CA proved to be a superior antioxidant compared with p-coumaric and ferulic acids, in inhibiting LDL oxidation and quenching of radicals and singlet oxygen (Gulçin, 2006). Chlorogenic acids and CA have an antioxidant potency in vitro and might inhibit the formation of mutagenic and carcinogenic N-nitroso compounds (Tapiero, Tew, Nguyen Ba, & Mathé, 2002). CGA and CA showed ability to inhibit N-nitrosation of an aromatic compound (2,3-diaminonaphthalene) via scavenging nitrogen sesquioxide (N_2O_3) faster than most of other antioxidants (Kono et al., 1997).

Nakatani et al. (2000) demonstrated that some CGA isomers (3-, 4- and 5-CQA) isolated from prune showed antioxidant activity, such as elimination of superoxide radicals and inhibitory effect against oxidation of methyl linoleate. Additionally, Natella et al. (2002) evaluated the antioxidant capacity of coffee and black tea and showed that coffee has a higher antioxidant capacity than tea in hydrophilic medium. Whereas both drinks have phenolic compounds with different capabilities antioxidant activities, substances possibly responsible for these effects would be CA in coffee and catechins in tea.

In the same way, oxidative injuries can also perturb the cellular energy homeostasis by disrupting the mitochondrial integrity. Reactive oxygen species (ROS) can induce permeabilization of the mitochondrial membrane resulting in the release of apoptosis initiating factors (AIF), such as cytochrome c, Smac/DIABLO and dissipation of the mitochondrial membrane potential (Clifford, 2000). Furthermore, increased levels of ROS induce the activation of capases-9 and -3, which will lead to apoptosis (Ashkenazi & Dixit, 1999).

Although the mechanism by which the hydroxycinnamic acids prevent oxidative stress are not yet fully elucidated, it seems to be mediated by translocation of Bad from the cytosol to the mitochondria where it dimerizes with Bcl-2 and Bcl-XL thereby neutralizing their mitochondrial stabilizing effect (Johnston, Clifford & Morgan, 2003). Chlorogenic acids may prevent oxidative stress induced mitochondrial transition pore complex opening by decreasing production of Bax and Bak protein, favoring an increase in Bcl2–BclXL/Bax–Bak ratio (Johnston et al. 2003).

In vivo animal models, the physiological relevance of CA through a direct contribution to the antioxidant defense system was demonstrated by Nardini et al. (1997). Dietary supplementation of CA (0.2 and 0.8% w/w) in rats resulted in a statistically significant increase of alfa-tocopherol both in plasma and in lipoprotein. Lipoproteins from caffeic acid-fed rats were more resistant than control to Cu2+catalyzed oxidation.

4. Role of Hydroxycinnamic Acids on Different Cancer Cells

Cancer is already the leading cause of death in many high-income countries and is set to become a major cause of morbidity and mortality in the next decades in every region of the world, in spite of the enormous amount of research and rapid developments that have proceeded in the past decade. Therefore, considerable attention has been focused on chemoprevention as an alternative approach to the control of cancer (Kang, Shin, Lee, & Lee, 2011).

Evidence that hydroxycinnamic acids may have a potential inhibitory effect on cancer invasion and metastasis is increasingly being reported in the scientific literature (Weng & Yen, 2012). During the last years many efforts have been made in order to elucidate the mechanisms involved by which hydroxycinnamic acids prevents cancer. These evidences are described below and summarized in Table 1. The possible mechanisms involved in anti-cancer effect of hydroxinamic acids is summarized in Figure 4 and we discuss depending on the site of cancer.

Table 1. Effects of hydroxycinnamic acids acids in different cancer cell lines and animal models

Cancer type	Cell/Animal model	Hydroxycinnamic acids and derivates	Anticarcinogenic activities	References
COLON	RKO and HT-29 colon cancer cell lines	3,4-, 3,5- and 4,5-diCQA fraction	(-) COX-2/PGE_2 and iNOS/NO pathways	Puangpraphant et al., 2011
COLON	RKO and HT-29 colon cancer cell lines	Caffeic acid	(-) matrix metalloproteinase-9 (MMP-9) activity	Puangpraphant et al., 2011
COLON	RKO and HT-29 colon cancer cell lines	DiCQA fraction	(-) proliferation of RKO and HT-29; ↑ ratio of Bax:Bcl-2 protein expression; ↑ Apoptosis	Puangpraphant et al., 2011
COLON	CT26 cells	Caffeic acid phenyl ester (CAPE)	(-) cell invasion; ↓ MMP-2/-9 and VEGF productions; ↓ pulmonary metastatic capacity	Weng & Yen, 2012
COLON	HCT116 cells	Caffeic acid phenyl ester (CAPE)	↑ G_0/G_1 phase cells, ↓ S phase ratio and ↑ apoptosis rate	Wang et al., 2005
COLON	Caco-2 cells	Ferulic acid (FA) and p-coumaric acid (p-CoA)	(-) cell cycle progression	Janicke et al., 2011
COLON	DLD-1 cell	diCQA from sweet potato leaf	↓ cancer cell proliferation	Kurata et al., 2007
COLON	SW480 cells	p-coumaric acid, caffeic acid, ferulic acid	↓ numbers of viable cells and colony formation	Hudson et al., 2000
GASTRIC	Kato III cell	diCQA from sweet potato leaf	↓ cancer cell proliferation	Kurata et al., 2007
GASTRIC	SNU638 and AGS cell lines	Ferulic acid (FA) and caffeic acid (CA)	Cytotoxic effects ↑ free radical scavenging	Kim et al., 2011
GASTRIC	Human gastric cancer	Caffeic acid phenyl ester (CAPE)	(-) MMP-9 expression (-) invasive capacity	Wu et al., 2007
LIVER	AH109A cell line	Chlorogenic acids	(-) invasive capacity	Weng & Yen, 2012
LIVER	Hep3B cells	Chlorogenic acids	(-) MMP-9	Weng & Yen, 2012
LIVER	AH109A cells	Caffeic acid	(-) invasion of AH109A cells in vitro	Weng & Yen, 2012
LIVER	HepG2 cells	Caffeic acid and caffeic acid phenyl ester (CAPE)	(-) PMA-induced MMP-9 expression; (-) binding activity of NF-κB	Weng & Yen, 2012
LIVER	HEPG2 cells	Caffeic acid and caffeic acid phenyl ester (CAPE)	(-) proliferation (dose-dependent); (-) MMP-9 expression	Jaganathan et al., 2009
LIVER	SKHep1 cells	Caffeic acid phenyl ester (CAPE)	(-) MMP-2 and -9 expressions	Weng & Yen, 2012
PROSTATE	DU145	Hydroxycinnamic acids	↑ apoptosis; ↑ cytotoxicity	Szliszka et al., 2011
PROSTATE	PC3 cells	Caffeic acid	(-) invasion of human prostate cancer cells	Weng & Yen, 2012

BREAST	MDA MB 468 and HBL 100 cells	P-coumaric acid, caffeic acid, ferulic acid,	↓ numbers of viable cells and colony formation	Hudson et al., 2000
BREAST	T47D cells	Caffeic acid and ferulic acid	(-) cell growth (time and dose-dependent)	Kampa et al., 2004
BREAST	T47D cells	Caffeic acid	↑ apoptosis via the Fas/FasL system	Kampa et al., 2004
LUNG	Tumor cell lines	Caffeic acid phenethyl ester (CAPE)	↓ tumor weight and number of tumor nodules	Nagaoka et al., 2003
LUNG	Tumor cell lines	CAPE and analogues	↓ tumor nodules in lung metastasis formation	Nagaoka et al., 2003
LUNG	Primary tumors(mice)	Caffeic acid	(-) proliferation, ↑ macrophage phagocytic activity and ↑gene expression and production of macrophage-related cytokines	Feng et al., 2010

Legend: (-): inhibition, suppression

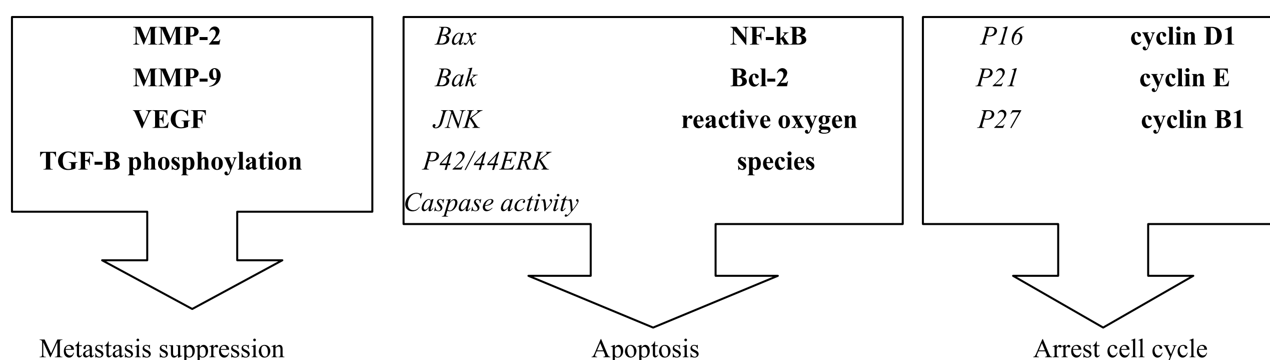

Figure 4. Potential mechanisms of anti-cancer activity of hydroxycinnamic acids

The signaling molecules being activated by hydroxycinnamic acids are shown in italic and those being suppressed by hydroxycinnamic acids are shown in bold

4.1 Colon Cancer

The colon cancer appears as the third most common type of cancer among men and the second among women. Studies have shown that the risk of developing colorectal cancer can be prevented by diet, with a decreased intake of dietary fat and an increased intake of cereal grains and dietary fiber (Parkin, Pisani & Ferlay, 1999). Inflammatory bowel disease is another risk factor for development of colon cancer (Yio & Itzkowitz, 2004). Inflammatory agents that suppress NF-kB or NF-kB-regulated products should have a potential in both the prevention and treatment of cancer. Nuclear factor kB (NF-kB) is a transcription factor and essential component link between inflammation and cancer. In innate immune pre-neoplastic and malignant cells, NF-kB upregulates inflammatory cytokines and enzymes including cyclooxygenase-2 (COX-2) and inducible nitric oxide (iNOS), which are an important factor for the synthesis of inflammatory mediators prostaglandin E2 (PGE2), anti-apoptosis, angiogenesis and metastasis (Puangpraphant, Berhow, Vermillion, Potts & Mejia, 2011).

Puangpraphant et al. (2011) demonstrated that 3,4-, 3,5- and 4,5diCQA fractions shown anti-inflammatory effect by suppressing the COX-2/PGE2 and iNOS/NO pathways. The diCQA fractions reduced nitric oxide (NO) by inhibiting iNOS enzyme. The capacity in reducing NO production of diCQA fraction was compared to CA. Besides this, CA showed an inhibitory effect of matrix metalloproteinase-9 (MMP-9) activity, which is known to be involved in tumor cell invasion and metastasis and anti-inflammatory activities. Additionally, it was shown that diCQA fractions inhibited proliferation of RKO and HT-29 human colon cancer cells. The diCQA fractions inhibited RKO and HT-29 cell proliferation by inducing apoptosis rather than arresting cell cycle. Apoptosis occurs through induction of the ratio of Bax:Bcl-2 protein expression and diCQA induced the cleavage of procaspase-3 to active caspase-3, which is a key step of apoptosis (Puangpraphant et al., 2011).

Invasion and metastasis are fundamental properties of malignant cancer cells. A number of proteolytic enzymes

participating in these processes, which involve degradation of environmental barriers such as the extracellular matrix (ECM) and the basement membrane. Weng & Yen (2012) showed that CT26 cells treated with caffeic acid phenyl ester (CAPE) exhibited not only cell invasion inhibition, but also a decrease in matrix metalloproteinase (MMP)-2/-9 and vascular endothelial growth factor (VEGF) productions. Intraperitoneal injection of CAPE into BALB/c mice reduced the pulmonary metastatic capacity of CT26 cells and decreased the plasma VEGF level.

On the other hand, when HCT116 cells were exposed to CAPE in different concentrations and times, CAPE displayed a strong growth inhibitory effect in a dose and time-dependent manner against HCT116 cells. Flow cytometry analysis showed that the ratio of G_0/G_1 phase cells increased, the S phase ratio decreased and apoptosis rate increased after HCT116 cells were exposed to CAPE (10, 5 and 2,5 mg/L) for 24 h (Wang et al., 2005).

Eberhardt et al. (2000) reported that apple extracts inhibited the proliferation of Caco-2 cells in dose-dependent manner. Additionally, McCann et al. (2007) demonstrated that the hydroxycinnamic acids components of apples were linked to inhibition of colon cancer in vitro. These phenolic compounds extracted from apples beneficially modulated three risk biomarkers of colorectal cancer in vitro without any cytotoxic effect. DNA damage was decreased (associated with tumor initiation), colonic barrier function was enhanced (associated with decreasing tumor promotion) and invasive potential was reduced (associated with reduced tumor metastatic potential).

Jaganathan et al. (2009) in your study indicated that phenylethyl caffeate (PEC), phenylethyl-3-methylcaffeate (PEMC), and phenylethyl dimethylcaffeate (PEDMC) present in the honey, inhibited azoxymethane (AOM)-induced colonic preneoplastic lesions, ornithine decarboxylase (ODC), tyrosine protein kinase (TPK), and lipoxygenase activity, which were relevant to the colon carcinogenesis. In addition, Janicke et al. (2011) investigated the effects of ferulic acid (FA) and *p*-coumaric acid (*p*-CoA) treatment on global gene expression in Caco-2 colon cancer cells. A total of 517 genes was significantly affected by FA and 901 by p-CoA. They found that FA or p-CoA treatment delayed cell cycle progression and the expressions of a number of genes involved in centrosome assembly, such as RABGAP1 and CEP2, were upregulated by FA treatment as well as the gene for the S phase checkpoint protein SMC1L1 (Janicke et al., 2011).

4.2 Gastric Cancer

Gastric cancer is the second highest cause of cancer-related death in men and the fourth among women (Kim et al., 2011). The etiology of gastric cancer involves Helicobacter pylori (H. pylori) infection, chronic active or atrophic gastritis and intestinal metaplasia. Integrated research in molecular pathology has shown that gastric cancer is a chronic proliferative disease characterized by multiple genetic and epigenetic alterations, i.e., a disease of altered gene expression.

H. pylori colonization may also activate nuclear factor-kappa B (NF-kB). Thus, NF-kB has become a good therapeutic target for gastric cancer and numerous efforts are being made to develop safe NF-kB inhibitors. Among hydroxycinnamic acids, the most hopeful is caffeic acid phenethyl ester (CAPE), which is a phenolic antioxidant structurally related to 3,4-dihydroxycinnamic acid, and can be obtained from propolis, a honey constituent. H. pylori infection enhanced the activity of NF-kB and the expressions of MMP-9, IL-1b, and IL-8 in gastric adenocarcinoma cell line. However, the augmented responses could be significantly reversed by CAPE treatment. In addition, in vitro studies showed that CAPE inhibits tumor growth and the capacity for invasion. (Ribeiro & Safatle-Ribeiro, 2007).

Nuclear expression of NF-kB was significantly more frequently observed in gastric cancer tissues than in nonmalignant gastric tissues. Wu et al. (2007) demonstrated that CAPE reduced MMP-9 expression induced by *H. pylori* and inhibited the invasive capacity of gastric cancer cells stimulated by IL-8 (inflammatory response) (Wu et al., 2007).

Recently, other natural products such as cinamic acids, coumarins and their components have been reported to have anti-*H.pylori* activity. Kim et al. (2011) investigated anti-gastritis activities of *Cimicifuga heracleifolia* (CH) ethanol extract which contains ferulic acid and caffeic acid. Their results showed that hydroxycinnamic acids exhibited higher free radical scavenging activity than other constituents and inhibited colonization of *H. pylori* effectively. In addition, the cytotoxic effects of CH ethanol extract and its constituents were investigated in SNU638 and AGS gastric cancer cell lines. The results showed that caffeic acid was more sensitive in AGS cells than in SNU638 cells, suggesting that this compound have a direct anti-cancer effect and protects against gastric injury induced by *H. pylori* (Kim et al., 2011). Additionally, Kurata et al. (2007) isolated diCQA from sweet potato leaf and showed that diCQA depressed Kato III (human stomach carcinoma cell line) cell growth in a dose-dependent manner.

4.3 Liver cancer

Epidemiological evidence suggests that moderate coffee consumption may help to reduce the risk of liver cancer, and the risk falls as coffee consumption rises. Literature indicates an inverse dose-dependent relationship between coffee consumption and the risk of hepatocellular carcinoma independent of its etiology (Gelatti et al., 2005).

Natella et al. (2002) demonstrated that the roasted coffee extract has phenolic compounds which are capable to increase the antioxidant activity in human plasma. Besides antioxidant activity of coffee constituents, studies show that polyphenols presented in coffee can activate the endogenous antioxidant system, leading to increased levels of hepatic glutathione, inhibition of lipid peroxidation and protection of rats liver from hepatotoxic action (Marsella, Di Benedetto, Vari, Filesi & Giovannini, 2005).

Chlorogenic acids, however, revealed an anti-invasive activity in rat ascite hepatoma cell line (AH109A) and inhibited MMP-9 expression in Hep3B cells (Weng & Yen, 2012). Additionally, CA demonstrated that suppresses invasion of AH109A cells in vitro. Treatment of HepG2 cells with CA and CAPE can suppress PMA-induced MMP-9 expression by inhibiting the binding activity of NF-κB. CAPE exerts anti-invasive potential through inhibition of MMP-2 and -9 expressions, possibly by targeting NF-κB in SKHep1 cells (Weng & Yen, 2012).

Jaganathan & Mandal (2009) showed that both CA and CAPE promoted a dose-dependent inhibitory effect on HepG2 cell proliferation. In HepG2 cells, CA (concentration of 200 μg/mL) reduced the cell viability to 61% compared to control, and treatment with CAPE (low concentration of 20 μg/mL) reduced the viability to 72% compared of control. CAPE and CA suppressed MMP-9 expression by blocking NF-κB activity. They also confirmed that CA (20 mg/kg) and CAPE (5mg/kg) repressed cell growth of tumor xenografts in nude mice as well as liver metastasis when administered subcutaneous or orally (Jaganathan & Mandal, 2009).

At the same time, Carrasco-Legleu et al. (2006) showed a protective effect of 2-phenylethyl 3(3,4-dihdroxyphenyl)-2-propenoate when a single dose was given before the initiation in rat hepatocarcinogenesis model. Administering CAPE in several doses during promotion caused a 90% decrease in the induction of c-glutamyl transpeptidase–positive (GGTþ) foci on day 25; decreases in markers of preneoplastic lesions, GGT activity, and the amount of glutathione-S-transferase class Pi (GST-p) protein were also observed. The results showed a protective effect against induction of preneoplasic lesions and are related to antioxidant and chemopreventive effects reported for CAPE (Weng & Yen, 2012).

4.4 Prostate Cancer

Chemoprevention of prostate cancer forms a three-pronged approach to reduce the burden of this common disease. Specifically, prevention aims to reduce disease incidence, thereby reducing the treatment-related side effects and potentially reduce disease-specific mortality. Xu & Chang (2012) showed that phenolic content presents in food legumes commonly consumed exhibited a significant linear correlation with antioxidant activities and also possessed strong cancer cell proliferation inhibitory effects in nine different cancer cell lines. The phenolic antioxidants in beans may reduce oxidative stress in vitro and in vivo, may also prevent carcinogenesis and inhibit cancer cell proliferation (Xu & Chang, 2012).

Szliszka et al. (2011) studied the action of ethanolic extract of propolis (EEP) and hydroxycinnamic acids in immunomodulatory, chemopreventive and antitumor effects in prostate cancer cells. It was observed cytotoxic and apoptotic activities of EEP against hormonesensitivity LNCaP and hormone-refractory DU145 prostate cancer cells. The results demonstrated that EEP and its components significantly sensitize to TRAIL-induced death in prostate cancer cells. The strongest cytotoxic effect on LNCaP cells was exhibited among other compounds by caffeic acid phenylethyl ester (CAPE).

Likewise, coffee may be associated with a reduced risk of prostate cancer. This contains many biological active compounds, including caffeine and chlorogenic acids that have potent antioxidant activity and can affect glucose metabolism and sex hormone levels. Wilsom et al. (2011) observed a weak inverse association between total coffee intake and incidence of prostate cancer. Men who consumed six or more cups per day had an 18% lower risk of prostate cancer compared with men who did not drink coffee.

Roles of insulin-like growth factors in prostate cancer biology are now well-established. Recent clinical and laboratory data support the hypothesis that insulin itself also influences the behavior of prostate cancer. Insulin levels have been associated with a greater risk of cancer progression or mortality among men diagnosed with prostate can-cer. Coffee contains CGA, which inhibit glucose absorption in the intestine and may favorably alter levels of gut hormones, which affect insulin response. Quinines, the roasting products of CGA, inhibit liver

glucose production in experimental models (Wilsom et al., 2011).

The role of caffeine and other components of coffee, regular and decaffeinated coffee separately, was investigated in prostate cancer. Similar associations with lethal and advanced can-cers were found for both (Wilson et al., 2011). Additionally, Weng & Yen (2012) demonstrated an effective inhibition of *in vitro* invasion of human prostate cancer cells (PC3) using CA treatment.

4.5 Breast Cancer

Antioxidant effects, steroid receptor binding, direct interaction with intracellular elements and signaling systems and, aryl hydrocarbon receptor (AhR) binding and modification of subsequent signaling pathways have been proposed as possible mechanisms for the mediation of the breast oncoprotective effect of hydroxycinnamic acids.

Eight hydroxycinnamic acids, including p-coumaric acid, caffeic acid, ferulic acid, sinapic acid, vanillic acid and methoxycinnamic acid, were identified in the extracts of two different types of rice (bran and brown extract). Bran extract decreased numbers of viable MDA-MB 468 and HBL 100 breast cells, colon-derived SW480 cells and human colonic epithelial cells. It also reduced colony formation of SW480 colon and MDA-MB 468 breast cells. CA (50 μM) decreased the number of viable cells in all cancer cell lines studied, except HBL 100 (Hudson et al., 2000).

At the same time, Kampa et al. (2004) studied the antiproliferative action of caffeic acid, syringic acid, sinapic acid, protocatechuic acid, ferulic acid and 3,4-dihydroxy-phenylacetic acid (PAA) on T47D human breast cancer cells. The results showed a time and dose-dependent inhibitory effect on cell growth among compounds: caffeic acid > ferulic acid = protocatechuic acid = PAA > sinapic acid = syringic acid. The antioxidative activity of these phenolic acids in T47D cells does not coincide with their inhibitory effect on tumoral proliferation. PAA induced an inhibition of nitric oxide synthase, while CA competes for binding and results in an inhibition of aryl hydrocarbon receptor-induced CYP1A1 enzyme, an enzyme induced by aryl hydrocarbon receptor activation. Both agents, however, induce apoptosis via the Fas/FasL system. These study show to note that necrotic cells were constantly low, indicating that these substances are not cytotoxic (Kampa et al., 2004).

Additionally, in MCF7 breast cancer cells, inhibitors of NO synthesis (*N*G-nitro-L-arginin methyl ester) and NO scavengers induced apoptosis, via a p53-associated pathway, while in T47D cells suppression of NO production triggers an induction of apoptosis via a FKHRL1 (FOXO3a) kinase pathway, independent of phosphoinositide 3-kinase-Akt and caspase-3 activation. In this study, phenolic acids exert a direct antiproliferative action (Kampa et al., 2004).

4.6 Lung Cancer

Lung cancer is the leading cause of cancer death in the world. Unfortunately, current therapy is still inadequate, and the 5-year survival rate for lung cancer remains poor. There is general agreement that the incidence of lung cancer is determined mainly by active cigarette smoking followed by occupational exposures. Epidemiological research has provided increasing evidence that dietary habits may play an important role in lung cancer etiology.

In this context, the consumption of an adequate diet rich in antioxidants are very important. Evidences have shown that ferulic acid (FA), a polyphenol very abundant in vegetables, acts as a potent antioxidant in vitro, due to its ability to scavenge free radicals. Furthermore, FA inhibited the expression and/or activity of cytotoxic enzymes including inducible nitric oxide synthase, caspases and cyclooxygenase-2. On this basis, FA has been proposed for the treatment of several age-related diseases such as cardiovascular diseases and cancer (Barone, Calabrese & Mancuso, 2008).

Sudheer et al. (2007) demonstrated that ferulic acid (FA), at a concentration ranging to10 from 150 uM, counteracted nicotine-induced lipid peroxidation and GSH depletion in rat lymphocytes. In the same study, nicotine has been shown to significantly impair the antioxidant cell defense system, but co-administration of FA (150 uM) counteracted the nicotine-induced decrease in superoxide dismutase catalase, glutathione peroxidase, vitamin A, E and C contents and this antioxidant effect was comparable to that elicited by N-acetylcysteine treatment.

On the other hand, Nagaoka et al. (2003) examined the antiproliferative activity of CAPE and its 20 analogues against six tumor cell lines. The oral administration of CAPE (5 mg/mice/d) for 7 d after tumor inoculation decreased the tumor weight and the number of tumor nodules in lung by 50% compared to the control. Besides CAPE, 4-phenylbutyl caffeate, 8-phenyl-7-octenyl caffeate, 2-cyclohexylethyl caffeate and n-octyl caffeate at an oral dose of 2 mg/mice/d caused a 55%, 43%, 55% and 35% reduction of the tumor nodules in their lung metastasis formation, respectively (Nagaoka et al., 2003).

Feng et al. (2010) studied the efficacy of Prunella extracts in the prevention and treatment of lung cancer. Prunella is a herb rich in bioactive compounds, including phenol acids (caffeic acid). The efficacy of Prunella extracts from different regions was compared in vitro and in vivo, and the TNF-α activity in serum of tumor-bearing mice were also evaluated. Prunella showed significant activity in the prevention and treatment of lung cancer through antiproliferation, regulation of tumor cell division cycle, promotion of apoptosis, antioxidative effects, immune regulation, antimutagenic effects, stimulation of macrophage phagocytic activity and induction of gene expression and production of macrophage-related cytokines (Feng et al., 2010).

5. Conclusion

As discussed in this review, the cytoprotective effect of hydroxycinnamic acids in many experimental systems is well established. However, the potential use of supplemental hydroxycinnamic acids in the therapy of age-related human pathologies is still to be confirmed. The main concerns derive from the pharmacokinetics of hydroxycinnamic acids and in particular its poor bioavailability and metabolic fate. The first question to be addressed in further clinical studies should be the following: after oral supplementation in humans, does hydroxycinnamic acids reach tissue concentrations similar to those which have been shown to be effective in preclinical studies? Another confounding issue is that many studies have not defined the primary cellular site of action of hydroxycinnamic acids, with reported activities potentially caused by interaction with upstream binding partners, regulatory kinases, and receptors. Despite the knowledge about the antioxidant potential presented by hydroxycinnamic acids is of utmost importance studying the action of these substances *in vivo*, because no data were found regarding absorption, bioavailability conditions physiological plasma concentration and ideal for their protective activity against free radicals and cancer, although it has been found a high antioxidant potential these compounds *in vitro*.

Authors' Contributions

LD, MCM and JT wrote the paper; all authors have read and approved the final manuscript.

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