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| <b>14. ABSTRACT</b><br><br>The research results we obtained in this Army Concept grant include the following novel findings. A) We showed that 2,4-disulfonyl-phenyl-tert-butyl-nitron (2,4-SPBN) acts as a weak inhibitor of the enzymatic activity of the extracellular endosulfatase Sulf2 when the enzyme is acting as an aryl sulfatase on the substrate 4-methylumbelliferyl-O-sulfate (4-MUS) and that 2,4-SPBN was much more effective enzyme inhibitor when Sulf2 acts upon its natural substrate the 6-O-sulfate ester of heparin sulfate. B) We also made the novel observation that Sulf2 activity was inactivated by hydrogen peroxide. C) We also showed that the phenyl sulfonyl anti-cancer agent suramin very potently inhibited Sulf2 enzymatic activity. D) Utilizing a Balb/c nude mouse model we showed that tumor growth of MCF-7 human breast cancer cells was significantly suppressed by administering 2,4-SPBN in the drinking water at 250 mg/kg at 35 days when experiments had to be stopped. These novel observations help us understand the anti-cancer activity of 2,4-SPBN and the potential role of Sulf2 inhibitors as anti-cancer agents and the potential for novel phenyl-sulfonyl agents as possible breast cancer therapeutics. |                         |                                        |                                   |                                                         |                                                   |
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# Army Concept Grant Final Report Narrative

## Introduction.....

This Army Concept grant had as its goal to test a novel concept which has broad implications regarding the possible treatment of breast cancers. The novel concept was to test if a synthetic antioxidant designated as SA-S<sub>2</sub> (to conceal its identity from the review panel) would inhibit the enzymatic activity of the relatively newly discovered extracellular enzyme Sulf2 and if SA-S<sub>2</sub> would also decrease breast cancer development in a nude mouse model. We can now reveal that the synthetic antioxidant SA-S<sub>2</sub> is 2,4-disulfonyl- $\alpha$ -phenyl-*tert*-butylnitone or 2,4-disulfonyl-PBN. Our results clearly show that our novel concept is valid in that SA-S<sub>2</sub> did inhibit Sulf2 and that it did show anti-cancer activity of the growth MCF-7 human breast cancer cells in a nude mouse model. The results will be detailed in a later section. In addition we made other novel observations which when taken together with the collective group of all the observations we have previously made in studying the anti-cancer activity of the PBN nitrones has helped us generate new novel and testable ideas for future research in breast cancer treatment. To take advantage of the new findings and new ideas, these were coalesced into a new Army Idea grant and recently submitted. In the **Body** section I present the research background in detail and the rationale for the experiments we conducted with the funds provided by the Army Concept grant.

## Body..... Scientific Background and Rationale for the studies

Background on Sulf2. The extracellular enzyme Sulf2 removes 6-O-sulfate groups on glucosamine from sub-regions of intact heparin. It has been demonstrated that the action of Sulf2 alters the binding of protein ligands to immobilized heparin [1]. Heparin/heparan sulfate proteoglycans (HSPGs) are a major constituent of the extracellular matrix on cell surfaces. HSPGs interact with many protein cellular signaling ligands that are important in cancer development including fibroblast growth factors (FGFs), vascular endothelial growth factor (VEGF) and various cytokines and chemokines. The specificity and strength of the interaction of these protein ligands with HSPGs is highly dependent upon the pattern of sulfation modifications within the glycosaminoglycan chains of the HSPGs. Recently several observations have implicated that the action of Edosulfatases and Aryl Sulfatases are important therapeutic targets in cancer development [1-4]. The action of Sulf2 as well as Sulf1 has been shown to be very important in some experimental models of cancer including breast cancer, pancreatic cancer, lung cancer, liver cancer and colon cancer as well as others. We considered it possible that the sulfonyl derivatives of PBN ( $\alpha$ -phenyl-*tert*-butylnitronone ie S-PBNs) act as inhibitors of Sulf-2 simply because the S-PBNs have sulfonyl groups and possess chemical properties similar to sulfates and importantly are not readily taken up by cells and hence reside in the extracellular domain where Sulf2 exists. This straight forward rationale is the main reason we wanted to test this basic concept in the Army Concept grant. The background scientific rationale is discussed in more detail below.

Sulfatase Enzymes and Sulf2. Sulfatase enzymes catalyze hydrolytic desulfonation of sulfate esters (CO-S) and sulfamates (CN-S) yielding HSO<sub>4</sub> as well as the alcohol and amino products, i.e. ROH and RNH<sub>2</sub>, from the original substrates ROS<sub>3</sub><sup>-</sup> and RN(H)SO<sub>3</sub><sup>-</sup> respectively [5]. The sulfatases belong to a class of enzymes that are highly conserved sequentially, structurally and mechanistically across the whole range of eukaryote and prokaryote species and have a unique active-site aldehyde residue,  $\alpha$ -formylglycine, which is installed post translationally [5]. Hanson et al in their recent review of the sulfatases list 15 separate human sulfatases [5]. They listed 7 different aryl sulfatases (A,B, C, D, E, F and G) which either reside in the lysosome, ER or golgi and 6 different sulfatases that reside in the lysosome which cleave sulfates from glucosamine, glucouronate, and heparin iduronate substrates as well as two separate endosulfatases (HSulf-1 and

HSulf-2) which reside in the extracellular matrix and act upon heparan sulfate [5]. The S-PBNs also reside in the extracellular medium because they are not readily transported into cells. The many different sulfatases, despite their differing wide range of natural substrates display promiscuity with small aromatic substrates such as p-nitrophenol sulfate, p-nitrocatechol sulfate and 4-methylumbelliferone sulfate [5]. It is also important to note that the activity of Sulf1 and Sulf2 toward the small aromatic substrate 4-methylumbelliferone sulfate is 10,000-20,000 fold higher than the activity toward the sulfates on heparan GLcNS [5]. For this reason and also considering the fact that the S-PBNs are phenyl sulfonyls and hence a possible substrate for sulfatases, we consider it a distinct possibility that the S-PBNs may act to inhibit Sulf1 and Sulf2 sulfatases that also exist in the extra cellular matrix.

The full length cDNA of two closely related extracellular heparin-degrading endosulfatases were recently characterized in mice and human which is sometimes designated as HSulf-1 and HSulf-2 [6] however in this report we will refer to HSulf-1 and HSulf-2 as Sulf1 and Sulf2 consistent with most of the scientific literature. Sulf1 is the human ortholog of the recently identified QSulf-1 which is expressed in several regions of the quail embryo. Both enzymes were shown to have aryl sulfatase activity using 4-methylumbelliferone and both were shown to have sulfatase activity on the C-6 position of glucosamine within specific sub-regions of intact heparin [6]. The action of recombinant Sulf2 on immobilized heparin and the resultant alteration of the binding of receptor proteins has recently been examined [1]. It was shown that Sulf2 acting upon heparin abolished the binding of VEGF, FGF-1 and the chemokines SDF-1 and SLC. It was further demonstrated that treating heparin that already had bound these receptor proteins caused their release. It was also shown that the estrogen-dependent breast cancer cells (MCF-7) released Sulf2 into the extracellular fluid and that this native enzyme acted in a similar way to that of recombinant Sulf2 [1].

Sulf1 and Sulf2 in Cancer. There is compelling evidence that these two extracellular endoglucoamine-6-sulfatases are involved in cancer development. Research reports in this area have been published in breast cancer [1, 4, 7], pancreatic cancer [2], head and neck squamous carcinoma [8], ovarian cancer [7] and in myeloma [9]. In the case of breast cancer data mining from published studies using serial analysis of gene expression (SAGE) analysis it was shown that mRNA Sulf2 SAGE tags per 100,000 increased from about 2 in normal breast tissue to 12 in ductal carcinoma in situ tissue to 32 in invasive ductal carcinoma tissue [4]. In this same study it was noted that HSulf-2 was also increased in CNS tumors and in colon cancer too. Utilizing human breast carcinoma cell lines, they also showed that Sulf2 mRNA was expressed and the enzymatically active protein was released into the culture medium. It should be noted that of the six cell lines studied only 3 lines (MCF-7, BT20 and BT549 lines) had Sulf2 protein in the medium whereas the (WT47D, MDA-MB-435 and MDA-MB-453 lines) did not [4]. Utilizing 2 mouse models of breast cancer they also demonstrated that mRNA of Sulf2 was up-regulated in carcinoma tissue and premalignant lesions but was undetectable in normal breast tissue. It was also noted the Sulf2 protein was localized to the epithelial cells of tumors. This is supportive of the angiogenesis role of this protein as was demonstrated by the angiogenesis brought about by recombinant Sulf2 in the chick chorioallantoic membrane assay [4]. In a follow-up study it was also shown that recombinant Sulf2 treatment of immobilized heparin prevented the binding of VEGF, FGF-1 and the chemokines SDF-1 and SLC. It was also noted that Sulf2 treatment of heparin released VEGF and FGF-1 proteins which were previously bound to it. These experiments suggest that the action of Sulf2 on the release of VEGF and other protein receptor factors may be the root cause of the increased angiogenesis mediated by this sulfatase. Even though several reports clearly implicate the importance of Sulf2 in breast cancer, the importance of Sulf1 in cancer development appears to be still in question.

Anti-cancer activity of the PBN-nitrones. PBN refers to  $\alpha$ -phenyl-*tert*-butylnitron. Our laboratory has studied the PBN-nitrones as anti-cancer agents for over 13 yrs and shown that the parent compound PBN has anti-cancer activity in 3 experimental cancer models: A) the dietary choline deficiency induced rat liver cancer model [10-14], B) the C6 rat glioma model [15], and C) the APC<sup>min/+</sup> mouse model of colon cancer [16]. In addition to PBN we have also tested 2,4-disulfonyl-PBN (designated as OKN-007 in this study) in the rat C6 model of glioma and have found it to have significant

anti-cancer activity [17]. This is very important for a potential anti-cancer treatment compound because the compound 2,4-disulfonyl-PBN has been taken through extensive human safety trials in its commercial development (known as NXY-059 in these studies) as a treatment for acute ischemic stroke and shown to be a safe compound but not effective as a stroke drug [13]. Therefore if 2,4-disulfonyl-PBN has anti-cancer activity it would have a much shorter path to development as a breast cancer treatment because of the extensive prior work in humans which that this compound was very safe in humans.

Summary and Rationale. Summarizing this scientific literature background section regarding the possible role of sulfonated PBN derivatives and their possible role in cancer development by acting as competitive inhibitors of Sulf2, this appears to be an important area to consider especially in breast cancer, pancreatic cancer, colon cancer, liver cancer and lung cancer. As noted above in the earlier sections, there are many reports already showing that Sulf2 enhanced expression is important in breast cancer development. Therefore going into this study we considered it highly likely that the sulfonated PBN derivatives would act as inhibitors of Sulf2. Since the sulfonated PBN derivatives are present in the extracellular medium where Sulf2 is acting to decrease the sulfation of heparin binding proteoglycans it seems very likely that this could be prevented by 2,4-disulfonyl-PBN and as such this would help explain its anti-cancer activity. Also of significant importance is the fact that if 2,4-disulfonyl-PBN had anti-cancer activity in breast cancer it would have a much shorter path to development of a breast cancer treatment because of the prior work in humans with this compound.

## **Body.....Research Objectives, abbreviated methods and Research Results**

Research Objectives. The following is the original stated objectives. We will test the innovative concept that a synthetic sulfate ester form of an antioxidant denoted SA-S<sub>2</sub> possesses anti-cancer activity by inhibiting the extracellular endo-sulfatase HSulf-2 in vivo and prevents breast cancer development in an experimental model dependent upon Wnt autocrine signaling. Abbreviated methods. In **objective 1** we will determine if 2,4-disulfonyl-PBN is a substrate for extracellular HSulf-2 and determine its competitiveness with the standard aryl sulfatase substrate 4-methylumbelliferyl-sulfate (4-MUS). In **objective 2** we will use a mouse xenograph model to determine if the growth of tumors formed by MCF-7 breast cancer cells growing in Estradiol pellet-implanted ovariectomized female BALB/c nude mice is inhibited by 2,4-disulfonyl-PBN administered to the mice at 250 mg/kg in the drinking water.

Research Results of Objective 1. The results of our research devoted to achieving objective 1 can be summarized as such:

A). We have shown that 2,4-disulfonyl-PBN acts to inhibit Sulf2 in the extracellular matrix of MCF-7 (see Figure 1) breast cancer cells as well as several other cancer cells by using the 4-methylumbelliferyl-sulfate (4-MUS) assay method. We saw significant competitive inhibition of 2,4-disulfonyl-PBN with 4MUS when the compound was added at 10mM and more so when it was added at 20mM. Although this is a high concentration of 2,4-disulfonyl-PBN the substrate 4-MUS concentration in the assay was high ie 10mM. These results represent the first time that a research group has found that a phenyl sulfonyl compound can inhibit the sulfatase activity of Sulf2.

B). We have synthesized <sup>35</sup>SO<sub>4</sub> radioactive labeled extracellular heparin sulfate as a the natural substrate for Sulf2 to determine if 2,4-disulfonyl-PBN will inhibit Sulf2 when it is acting upon its natural substrate. We have shown that this is true and in this case Sulf2 activity is much more sensitive to 2,4-disulfonyl-PBN than in the case of 4-MUS as the substrate (see Figure 6 and compare to Figure 1). This is a very important observation suggesting that the anti-cancer activity of 2,4-disulfonyl could possibly be explained in part by this mechanism. These studies are still ongoing for this is a major finding of importance.

C). We found that another phenyl sulfonyl compound Suramin much more potently inhibits the activity of Sulf2 that does 2,4-disulfonyl-PBN. Suramin has 6 sulfonyl groups whereas 2,4-disulfonyl-PBN only has 2 sulfonyl groups (see Figures 4 and 5). Suramin does have anti-cancer activity also. This observation in a sense validates the fact that phenyl-sulfonyl are active against Sulf2.

D). We discovered that the Sulf2 enzymatic activity was inhibited by hydrogen peroxide thus indicating that an oxidative event caused inactivation of Sulf2 (see Figure 2 and 3). This has never been reported before and the significance of this observation is not known yet.

**Research Results of Objective 2.** The results of our research devoted to achieving object 2 can be summarized as such:

A). We set up and conducted a study where MCF-7 breast cancer cells were grown in the flanks of female ovariectomized BALB/c nude mice where one group of 20 animals were administered 250mg/kg 2,4-disulfonyl-PBN in their drinking water and the control group of 20 mice received only drinking water. The experiment started excellently and continued for 5 weeks. Tumor volumes were measured at 4 weeks and 5 weeks. At the 5 weeks time point the average tumor volume for each tumor of the untreated group was 309.16 +/-20.07 (SE) and the 2,4-disulfonyl-PBN treated was 208.6 +/-17.51 mm<sup>3</sup> respectively (see Figure 7). Therefore the average tumor volume was significantly different at 67% as large as the untreated tumors in the mice. The tumor volumes at 4 weeks were smaller in each group with the treated group being 90.19% of the volume of the control group. The difference between the two groups was statistically significant at the 4<sup>th</sup> week also even though not as impressive as at the 5<sup>th</sup> week. The data is presented in the attached figure( ). Therefore at this time in the experiment we were please to see the differences between the treated and untreated begin to increase as we had seen in the C6 glioma models where 2,4-disulponyl-PBN showed significant anti-cancer activity when given in the drinking water [17].

However it was between the 5<sup>th</sup> and the 6<sup>th</sup> week in the nude mouse study that a significant problem developed which made it necessary to abort the experiment. The problem was not caused by the PI or the scientific team working on the experiment but due to an animal husbandry error of the OMRF Laboratory Animal Research Center. The nude mice were not feed over a 2-3 day period and this caused several of them to die therefore the experiment had to be aborted. This type of mistake by the OMRF LARC has been extremely rare if ever happened before. The mistake was done in the time interval when the head Vet in charge of the LARC had resigned and the replacement head Vet had not started. The OMRF took full responsibility and will pay all costs for conducting the nude mouse study again to completion. The mistake was reported to the Army program officer in charge of this Concept grant and also to the NIH where many NIH-funded projects are being conducted. The PI and Dr Chandru will commence the nude experiment very soon and hope to have it completed before Jan 2011.

### **Key Research Accomplishments.....**

A). We showed that 2,4-disulfonyl-PBN acts to inhibit Sulf2 in the extracellular matrix of MCF-7 breast cancer cells as well as several other cancer cells by using the 4-methylumbelliferyl-sulfate (4-MUS) assay method.

B). We synthesized the <sup>35</sup>SO<sub>4</sub> radioactive labeled extracellular heparin sulfate as a the natural substrate for Sulf2 and showed that 2,4-disulfonyl-PBN inhibited Sulf2 when it was acting upon its natural substrate and that the effectiveness of 2,4-disulfonyl-PBN to Sulf2 acting upon its natural substrate was much more potent than to Sulf2 acting upon 4-MUS as its substrate.

C). We found that another phenyl sulfonyl compound Suramin much more potently inhibits the activity of Sulf2 that does 2,4-disulfonyl-PBN when Sulf2 is acting upon 4-MUS as a substrate.

- D). We discovered that the Sulf2 enzymatic activity was inhibited by hydrogen peroxide thus indicating that an oxidative event caused inactivation of Sulf2.
- E). We showed that 2,4-disulfonyl-PBN when administered at the rate of 250mg/kg to female ovariectomized BALB/c nude mice which were growing MCF-7 breast cancers had anti-cancer activity in a partially completed experiment.

### **Reportable Outcomes.....**

All 5 of the key research accomplishments are reportable outcomes. That is, they are research results that have never been reported before. It is however necessary to complete a few more experiments in research accomplishment D) and the nude mouse experiment E) must be done again and it will be done and then all of the results listed in research accomplishments A), B), C), D), and E) will be incorporated in a manuscript and submitted for publication. Therefore the reportable outcomes are listed below.

- A). We showed that 2,4-disulfonyl-PBN acts to inhibit Sulf2 in the extracellular matrix of MCF-7 breast cancer cells as well as several other cancer cells by using the 4-methylumbelliferyl-sulfate (4-MUS) assay method.
- B). We synthesized the  $^{35}\text{SO}_4$  radioactive labeled extracellular heparin sulfate as a the natural substrate for Sulf2 and showed that 2,4-disulfonyl-PBN inhibited Sulf2 when it was acting upon its natural substrate and that the effectiveness of 2,4-disulfonyl-PBN to Sulf2 acting upon its natural substrate was much more potent than to Sulf2 acting upon 4-MUS as its substrate.
- C). We found that another phenyl sulfonyl compound Suramin much more potently inhibits the activity of Sulf2 that does 2,4-disulfonyl-PBN when Sulf2 is acting upon 4-MUS as a substrate.
- D). We discovered that the Sulf2 enzymatic activity was inhibited by hydrogen peroxide thus indicating that an oxidative event caused inactivation of Sulf2.
- E). We showed that 2,4-disulfonyl-PBN when administered at the rate of 250mg/kg to female ovariectomized BALB/c nude mice which were growing MCF-7 breast cancers had anti-cancer activity in a partially completed experiment.

### **Conclusion....**

Utilizing the funds provided by the Army Concept grant we were able to test the notion that a phenyl-sulfonyl compound namely 2,4-disulfonyl-PBN, which we have shown to have anti-cancer activity, will act to inhibit the enzymatic activity of Sulf2 and to act as an anti-cancer agent in a Xenograph model of breast cancer. Our results have clearly shown that 2,4-disulfonyl-PBN does inhibit the activity of Sulf2 when the enzyme is using 4-MUS as the substrate and to more effectively inhibit the enzyme when it is using heparin its natural substrate. These are novel observations from several standpoints; first no one has ever shown that a phenyl-sulfonyl compound will inhibit Sulf2 before, second no one has ever tested 2,4-disulfonyl-PBN as an inhibitor of Sulf2 before and third this observation may be very important in explaining the anti-cancer activity of 2,4-disulfonyl-PBN. With respect to testing the anti-cancer activity of 2,4-disulfonyl-PBN in a Xenograph breast cancer model our preliminary (yet incomplete) studies clearly implicates that this is the case. We also made two other novel observations on the Sulf2 enzyme namely; A) that hydrogen peroxide inhibits its activity implicating that its activity is sensitive to oxidative damage and B) that suramin, a known anti-cancer agent [18-23], potently inhibits the Sulf2 enzyme. Once we have completed the Xenograph model study and also finished up the biochemical studies the novel results will be submitted to a quality scientific journal specializing in publishing cancer research results. We already have presented the existing research results arising from this Concept grant at two major scientific meetings; namely the Society of Free Radical Biology and Medicine meeting in San Francisco Nov 2009 and the American Association Cancer Research in Washington DC April 2010 where it was received with considerable interest. The abstracts from these two meetings are attached in the appendices.

Another unexpected benefit has arisen from the conduct of the experiments funded by the Army Concept grant. The ability to focus intently upon the cancer enhancing action of Sulf2 has helped us to make an unexpected connection to our past research on the anti-cancer activity of the PBN nitrones. It has been shown that Sulf2 acting upon heparin enhances the mobility of SDF-1 (Stroma-derived factor 1)[1]. We realized recently that SDF-1 plays an important role in the action of its cellular receptor CXCR4 to set up a CXCR4/SDF-1 gradient that is very important in causing mesenchymal stem cells (MSCs) in the blood to home to and become incorporated into the growing primary tumor as cancer-associated fibroblasts, i.e. CAFs[24-26]. Recent research has shown that within the tumor, cancer cells and CAFs form a paracrine signaling network that enhances tumor growth. Nitric Oxide (NO) is a free radical signaling agent formed in most tumors and through unknown mechanisms enhances tumor growth [14]. It has more recently come to light that NO mediates the enhanced expression of CXCR4 in tumor cells and in stem cells [27-30]. One of the primary observations that our past research has shown is that the PBN nitrones suppresses the expression of inducible nitric oxide synthase (iNOS) which is responsible for producing significant levels of NO [14]. Therefore focusing on Sulf2 has allowed us to realize that the anti-cancer action of PBN nitrones in suppressing iNOS expression in tumors most likely suppresses tumor growth by suppressing homing of MSCs to the growing primary tumor and in the case of 2,4-disulfonyl-PBN by suppressing the action of Sulf2 which suppresses the mobility of SDF-1 and thus suppresses the CXCR4/SDF-1 gradient leading to decreased homing of MSCs to the primary tumor. It should be noted that in the case of the rat C6 model of glioma we have shown that 2,4-disulfonyl-PBN does decrease iNOS expression [17]. Therefore this exciting new realization of the synergistic connection between our results obtained in this Army Concept grant with that of our past results has been incorporated into a new Army Idea grant submitted in April 2010. This is a hot new area of research that needs to be explored in more depth. We are thankful to have been given the opportunity to investigate the action of 2,4-disulfonyl-PBN on Sulf2 and to begin to more fully explore its anti-cancer action as a potential treatment for breast cancer.

## References.....

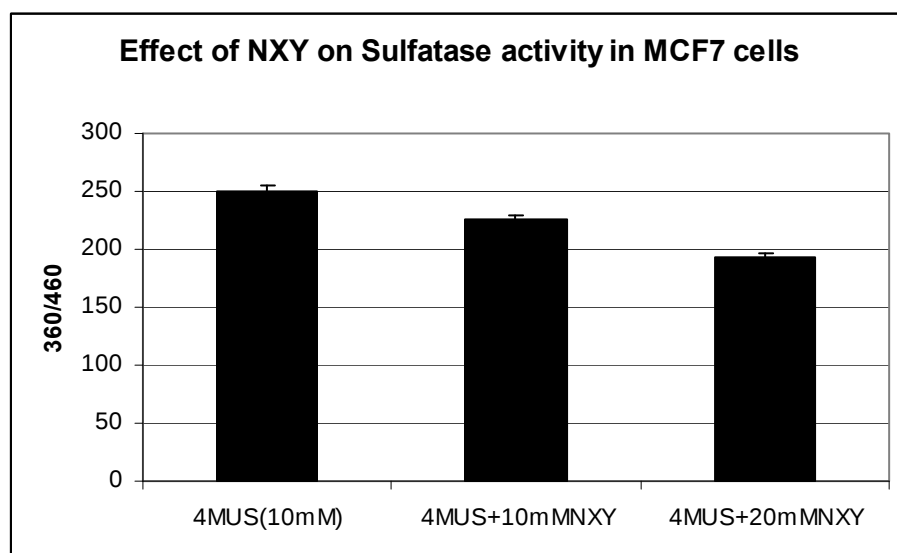
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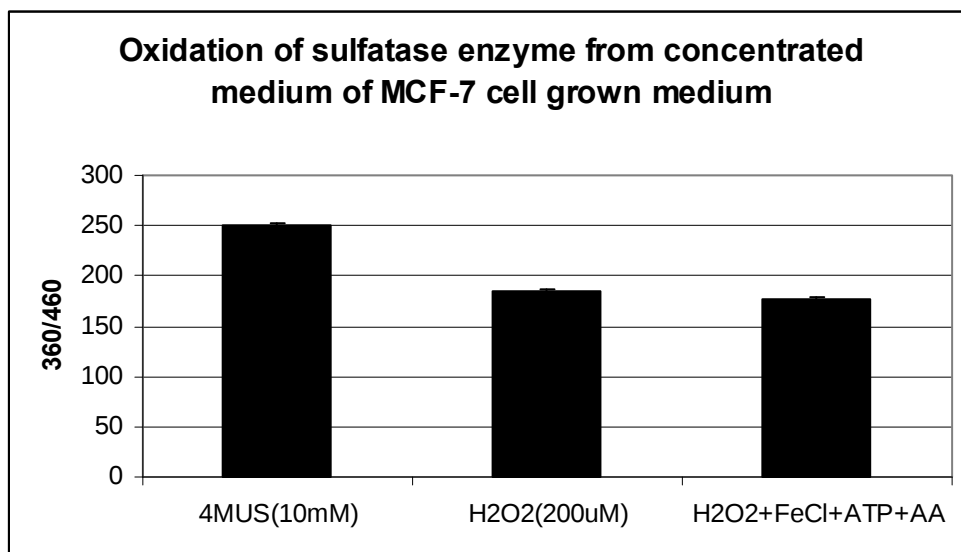
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#### Appended Figures and Figure Legends.....

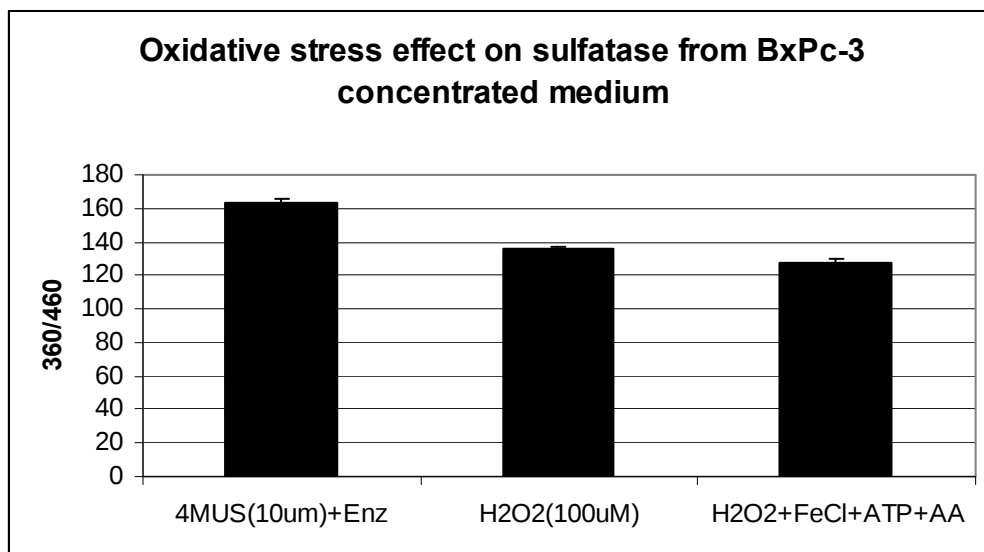


**Figure 1.** Sulfatase enzyme was obtained from the culture media of MCF-7 human breast cancer cells which were grown in DMEM medium containing 10% FBS for 3 days. Dead cells from the medium were separated by centrifuging at 2500rpm for 10 min and the medium concentrated using Millipore centurion tubes.

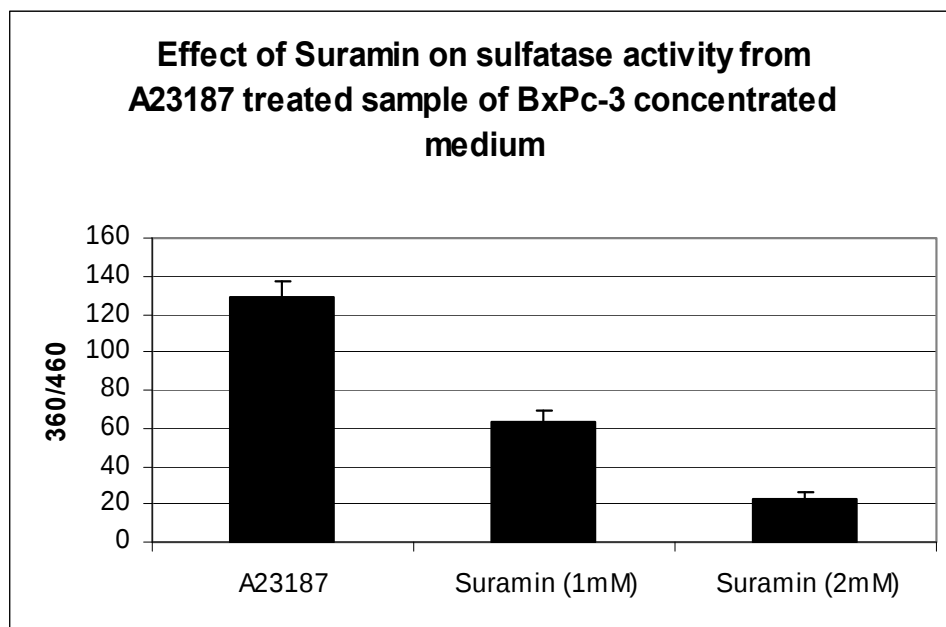
Concentrated medium was used as the source of Sulf2. The Sulf2 activity was assessed using 10mM 4-MUS and the influence of 10mM and 20mM 2,4-disulfonyl-PBN (NXY) in the presence of 10mM 4-MUS determined.



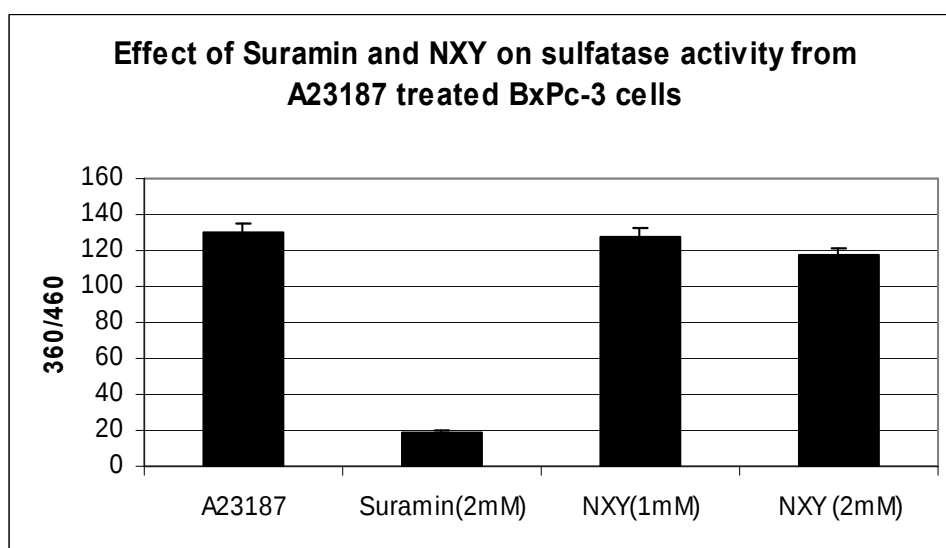
**Figure 2.** The Sulf2 enzyme present in the concentrated medium of MCF-7 cells was treated with either 200 $\mu$ M  $H_2O_2$  or 200 $\mu$ M  $H_2O_2$  plus ATP and chelated FeCl plus ascorbic acid (AA). The activity was determined in the presence of 10mM 4-MUS before or after oxidation with  $H_2O_2$  or  $H_2O_2$  plus FeCl/ATP/AA respectively.



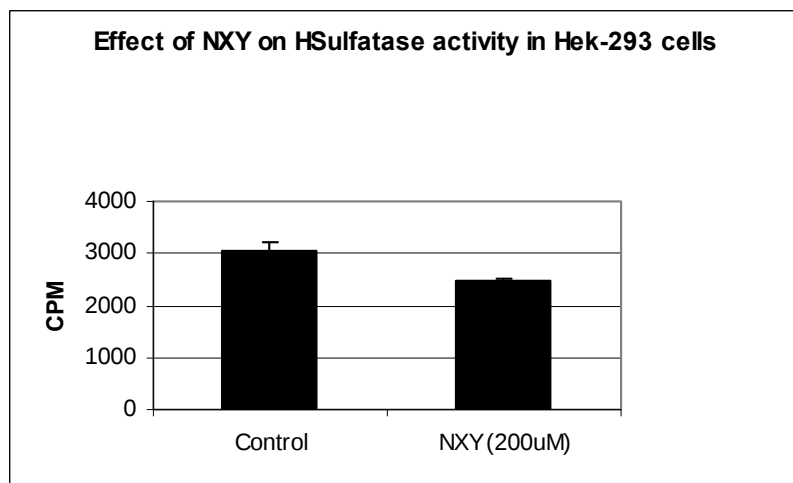
**Figure 3.** The Sulf2 enzyme present in the concentrated medium of BxPc-3 pancreatic cancer cells was determined using 10mM 4-MUS as substrate. The influence of enzyme oxidation using  $H_2O_2$  as well as  $H_2O_2$  plus FeCl/ATP/ascorbic acid was determined.



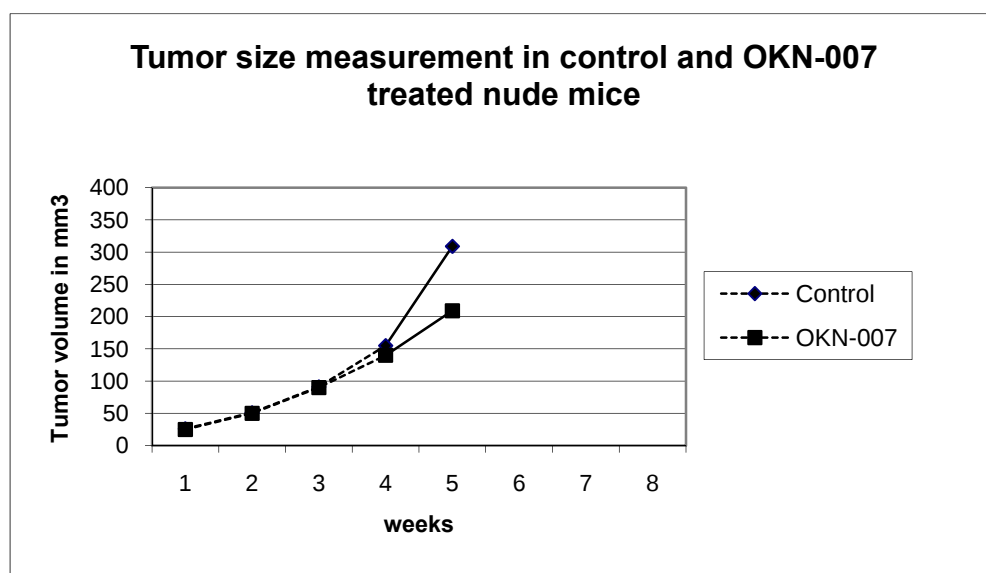
**Figure 4.** The Sulf2 enzyme present in the concentrated medium of BxPc-3 pancreatic cancer cells which had been treated with A2317 was used to a source of enzyme. The Sulf2 activity was assessed using 10mM 4-MUS as substrate and the influence of suramin at 1mM or 2mM was determined.



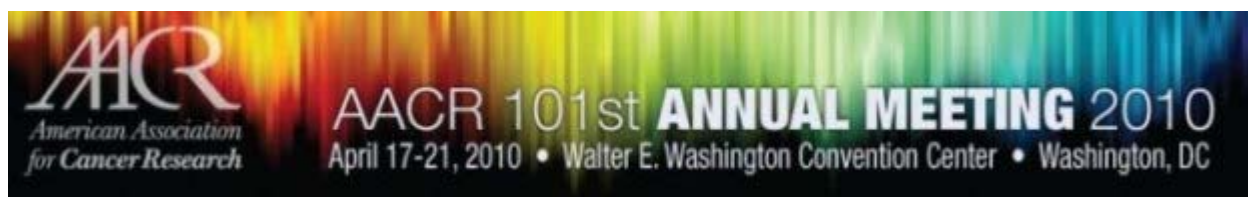
**Figure 5.** The Sulf2 enzyme in the concentrated medium as in Figure 4 was used. The Sulf2 activity was assessed as in Figure 4 and the influence of suramin (2mM) as well as 2,4-disulfonyl-PBN (NXY) at 1mM and 2mM was determined.



**Figure 6.** Sulf2 enzyme from Hek-293 (human embryonic kidney) cells transfected with Sulf2 plasmid was used. The  $^{35}\text{SO}_4$  labeled heparin natural substrate was used and the influence of adding 200 $\mu\text{M}$  2,4-disulfonyl-PBN (NXY) on the enzymatic activity was determined.



**Figure 7.** Presentation of the average MCF-7 tumor volumes as influenced by 2,4-disulfonyl-PBN (OKN-07) administration. The data points at week 4 and week 5 are actual values we obtained. The values at weeks 1,2 and 3 are not measured numbers but were inserted as estimates of the tumor volumes at those dates for illustration purposes only. At week 5 there is a significant difference in the average tumor volumes. There were 18 animals in the control group and the average tumor volume was  $309.17 \pm 20.07$  (SE) and N=18 whereas averaged tumor volume of the treated animals was  $208.6 \pm 17.51$  (SE) and N=20.



**Category: Tumor Biology 19**

**Session Title: Drug Targets, Angiogenesis, and Gene Networks**

**#2267 Inhibition of extracellular sulfatase 2 as a possible mechanism to partially explain the anticancer activity of the nitrone OKN007.** Hema K. Chandru, Rheal A. Towner, Robert A. Floyd. Oklahoma Medical Research Foundation, Oklahoma City, OK .

Sulfatase 2 (Sulf2) is an extracellular enzyme that has endosulfatase activity and catalyzes the removal of 6-O-sulfate groups on glucosamine from subregions of the glycosaminoglycans heparin sulfate. The action of Sulf2 has been shown to alter the binding of protein ligands to heparin which is involved in the binding of growth factors such as VEGF, FGF-2 and TGF to cellular receptors. Studies in experimental models have shown that fully active Sulf2 is important in the development of breast cancer and pancreatic cancer. Several tumors have been shown to be enriched in Sulf2 in comparison to the normal tissues where its level is either absent or present at very low levels. Sulf2 acts as a potentiator for the Wnt signaling and this is considered important in cancer stem cell growth. We have shown that PBN (alpha-phenyl-tert-butyl nitrone) has anti-cancer activity in three experimental models, A) the choline deficiency induced liver cancer model, B) the APC<sup>min</sup> model of colon cancer and C) the rat C6 glioma model. We have also shown that 2,4-disulfonyl-alpha-phenyl-tert-butyl nitrone (OKN007) has potent anti-cancer activity in the rat C6 glioma model. We considered based on the presence of phenyl sulfonyl groups that the nitrone 2,4-disulfonyl-alpha-phenyl-tert-butyl nitrone (OKN007) may act as a competitive inhibitor of Sulf2.

We have found that Sulf2 is secreted into the media of Sulf2 transfected Hek-293 cells as well as several cancer cells, including from the highest to lower levels; MCF-7 breast cancer cells, Hek-293 cells, C6 glioma cells and BxPc-3 pancreatic cancer cells. Utilizing the concentrated media of these cells we have shown that Sulf2 has aryl sulfatase activity against the sulfate ester of 4-methylumbelliferone. We have shown that phenyl sulfonyl compounds suppress Sulf2 activity in an apparent competitive action. For instance, we found that the polysulfonated compound suramin potently suppressed the Sulf2 aryl sulfatase activity and that 2,4-disulfonylphenyl-tert-butyl nitrone inhibited Sulf2 activity but less so than suramin. Utilizing concentrated extracellular media of several human cancer cell lines we have shown that Sulf2 activity is decreased by incubation with H<sub>2</sub>O<sub>2</sub>. Our results suggest that the anti-cancer activity of OKN007 may in part be explained by its inhibitory action against Sulf1. Research to test this concept is now underway.

#### **Citation Format**

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## **Sulfatase 2 Inactivation – Possible Role in Cancer Development**

*Robert Floyd<sup>1</sup>, Hema Chandru<sup>1</sup>, and Rheal Towner<sup>1</sup>*

*<sup>1</sup>Oklahoma Medical Research Foundation*

Sulfatase 2 Inactivation – Possible Role in Cancer Development

Hema Chandru, Rheal Towner and Robert A. Floyd

Sulfatase 2 (Sulf2) is an extracellular enzyme that has endosulfatase activity and catalyzes the removal of 6-O-sulfate groups on glucosamine from subregions of the glycosaminoglycans heparin sulfate. The action of Sulf2 has been shown to alter the binding of protein ligands to heparin which is involved in the binding of growth factors such as VEGF, FGF-2 and TGF to cellular receptors. Studies in experimental models have shown that fully active Sulf2 is important in the development of breast cancer and pancreatic cancer. Sulf2 has aryl sulfatase activity against the sulfate ester of 4-methylumbelliferone. Utilizing concentrated extracellular media of several human cancer cell lines we have shown that Sulf2 activity is decreased by incubation with  $H_2O_2$ . We have also shown that phenyl sulfonyl compounds suppress Sulf2 activity in an apparent competitive action. The polysulfonated compound suramin potently suppressed the Sulf2 aryl sulfatase activity and that 2,4-disulfonylphenyl-tert-butyl nitron inhibited Sulf2 activity but much less so than suramin. Research effort is proceeding on purifying the enzyme and characterizing the specific protein changes that occurs in  $H_2O_2$ -mediated inactivation as well as the inhibitory activity of sulfonyl compounds on Sulf2 activity against the glycosaminoglycan natural substrates.