



## Increasing Oxygen in Hypoxic Tumors

John L. Gainer<sup>1,2\*</sup> and Miles F. Lankford<sup>3</sup>

<sup>1</sup>Chief Scientific Consultant, Diffusion Pharmaceuticals LLC

<sup>2</sup>Department of Chemical Engineering (Emeritus), University of Virginia

<sup>3</sup>Molecular Assessments and Preclinical Studies (MAPS) Core, University of Virginia, Charlottesville, VA

### Abstract

**Background:** Tumors are frequently hypoxic, affecting both chemotherapy and radiotherapy. Trans sodium crocetinate (TSC), a novel pharmaceutical agent, causes increases in oxygen levels of hypoxic tissues. It has also been shown, in animal models of cancer, that radiotherapy is more effective when used in conjunction with TSC.

An increase in tumor oxygen should also affect the HIF-1 $\alpha$  pathway. Thus, an *in vitro* study of that pathway in human glioblastoma cells was performed.

**Methods:** This study involved the use of quantitative real time polymerase chain reaction technology and human glioblastoma multiforme cells. The cells were cultured under both hypoxic and normoxic conditions.

**Results:** The inclusion of TSC in the media of the cells resulted in some genes in the HIF-1 $\alpha$  pathway being either up- or down-regulated in a statistically-significant manner. These changes were opposite to those which occurred when the same cells were grown under hypoxic conditions but without TSC. In addition, those same genes reacted in an opposite manner when the cells were grown with TSC but under a normal oxygen environment.

**Conclusions:** These results support previous observations that TSC reduces hypoxia in tumor cells. Since TSC caused statistical differences in gene expression under hypoxia different from those caused under normoxia, it suggests that there is not a direct effect of TSC on the HIF-1 $\alpha$  pathway. Rather, TSC alters the gene expression due to a change in the response of the genes to different oxygen levels. These data also correlate with previous *in vivo* studies which show that TSC increases oxygen to hypoxic tissue but not to normal tissue.

Thus, these data, combined with previous studies of animal cancer models, strongly suggest that TSC has the ability to increase cellular oxygen in tumor cells. Such a physiological change can be beneficial with combined with radiotherapy for cancer.

**Keywords:** Tumor; Trans sodium crocetinate; Hypoxia; Oxygen; Radiosensitizer; Radiotherapy; HIF-1 $\alpha$

### Introduction

Trans sodium crocetinate (TSC) is a novel pharmaceutical agent which increases the oxygen transport to hypoxic tissue. Many solid tumors are hypoxic, and it has been suggested that increasing tumor oxygen levels could enhance the effectiveness of both radio- and chemo- therapy [1].

TSC acts systemically, and was specifically developed to treat hemorrhagic shock. For example, it is shown to increase whole-body oxygen consumption in rats to nearly normal values even after a 50 – 60% blood loss [2]. TSC is proposed to increase the diffusion of oxygen from the red blood cells to hypoxic tissues by causing an increase in the amount of hydrogen-bonding among the water molecules of the blood plasma [3].

Sheehan and coworkers in the Department of Neurosurgery at the University of Virginia have published several manuscripts dealing with the use of TSC to treat a rat model of glioblastoma multiforme. They injected C6 cells into rat brains in order to form tumors there. They have then shown that TSC enhances the oxygen levels in the tumor both by using an oxygen electrode [4] as well as by using PET imaging [5]. In contrast, TSC does not affect the oxygen tension of normal brain tissue [4]. They have also shown that the combination of TSC and radiation can result in a tripling of survival of the cancerous rats as compared to those which receive radiation or radiation/temozolomide alone [6,7].

In a human study, TSC had been shown to both reduce the onset time of pain and to increase the peak walking time in patients having

severe peripheral artery disease [8], both of which have been linked to tissue hypoxia [9]. A Phase 1/2 oncology clinical trial will begin soon using TSC in conjunction with radiation and temozolomide for the treatment of newly-diagnosed glioblastoma multiforme (clinicaltrials.gov identifier: NCT01465347). It is hoped that the TSC will enhance the tumor oxygen levels in these patients so that the radiation can be more effective.

Undoubtedly, the development of a new, easily implemented way to augment cellular oxygen levels could be extremely beneficial, especially for radiotherapy of cancerous tumors. Although the mechanism of action for TSC does not rely on receptors or genetic alterations, it might be expected that augmenting cellular oxygen levels would affect the HIF-1 $\alpha$  genetic pathway. Thus, the effects of TSC on that pathway are reported here.

### Materials and Methods

The effects of TSC on the HIF-1 $\alpha$  pathway in hypoxic and normoxic U-87 (human glioblastoma) cells were measured using quantitative real

**\*Corresponding author:** J. L. Gainer, Chief Scientific Consultant, Diffusion Pharmaceuticals LLC, 2020 Avon Court, #4, Charlottesville, VA 22902, USA, Tel: 434-220-0718; Fax: 434-220-0722, E-mail: [jgainer@diffusionpharma.com](mailto:jgainer@diffusionpharma.com)

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time polymerase chain reaction technology (qRT-PCR). The cell line was obtained from the American Type Culture Collection (ATCC), Manassas, VA. The qRT-PCR kit was obtained from SABiosciences, Frederick, MD.

### Cell culture

Cells were grown in minimum essential media (MEM) supplemented with 10% fetal bovine serum (FBS) and non-essential amino acids (NEAA) from Life Sciences, Grand Island, NY. Following 2 passages, cells were plated in 100 mm dishes at a cell density of  $1.0 \times 10^6$  cells/mL.

### Treatment

Cells were grown overnight, and at time of treatment were approximately 70% confluent (cells were not packed or overlapping). The original medium was removed and replaced with medium + TSC (the concentration of TSC was 3.3  $\mu\text{g/mL}$  of medium), as well as plain medium for the control cells.

The cells treated under normal oxygen conditions (normoxic) were placed in a  $\text{CO}_2$  incubator (95% air, 5%  $\text{CO}_2$ ). Cells treated under hypoxic conditions were placed in a special chamber (Billups-Rothenberg Modular Incubation Chamber, Del Mar, CA) which was purged for 10 min with a mixture of 5%  $\text{CO}_2$ , 25%  $\text{O}_2$  and the remainder nitrogen. Then, the chamber was quickly sealed and placed in an incubator at 37 °C. Cells were cultured for 20 hrs.

### RNA

All cells were lysed in 600  $\mu\text{L}$  of lyses buffer supplied with the kit. RNA was purified using an RNA-easy kit (Qiagen, Valencia, CA). From the purified RNA, c-DNA was synthesized using a first strand RNA kit (Qiagen). Each resulting c-DNA was added to RT2 Master Mix (SABiosciences), and loaded onto HIF-1 $\alpha$  human hypoxia pathway signaling plates and analyzed using an ABI Prism® 7900HT (Applied Biosystems, Carlsbad, CA).

### Data

The data were analyzed using the SABiosciences web-based analyses spreadsheet.

### Results

When adding TSC to the medium of cells cultured under hypoxic conditions, there were some statistically-significant changes noted in the gene expression of the HIF-1 $\alpha$  pathway. Genes that were statistically up-regulated (about 200%) with TSC + hypoxia are shown in Table 1 and those that were statistically down-regulated (about 40%) with TSC + hypoxia are shown in Table 2.

### Discussion

From Table 1, it is shown that 5 genes are up-regulated in a statistically-significant manner with hypoxia plus the addition of TSC to the cellular medium. Interesting enough, all of these same genes are down-regulated if TSC is not added to the media.

From Table 2, it is shown that 2 genes are down-regulated in a statistically significant manner with hypoxia and the addition of TSC. Once again, TSC causes opposite changes to those genes in the hypoxic control cells.

Tables 3 and 4 compare the normoxic changes found for the genes which were statistically up- or down-regulated under hypoxia. As can

GENE	Function	Non-TSC Response to Hypoxia
GPX1	Codes for a member of the glutathione peroxidase family.	Slightly down-regulated
MYBL2	Codes for a protein involved in cell cycle progression.	Slightly down-regulated
NOTCH 1	Codes for notch protein, which is involved in cell signaling.	Down-regulated
CSTB	Codes for protein cystatin-B.	Down-regulated
SUMO 2	Codes for a protein involved in a variety of cellular processes	Slightly down-regulated

**Table 1:** Genes Up-Regulated with TSC + Hypoxia (each approximately a 200% increase).

GENE	Function	Non-TSC Response to Hypoxia
VEGF A	Modulates the growth of new blood vessels.	Up-regulated
B2M	Codes for a protein found on nearly all cells having a nucleus.	Stable with hypoxia

When adding TSC to the media of the same U87 cells grown under normal oxygen conditions (normoxia), the genes listed in Tables 1 and 2 show opposite changes to those which occurred under hypoxic conditions. Tables 3 and 4 compare the hypoxic versus the normoxic behavior for the genes listed in Tables 1 and 2, and an asterisk denotes when the change was statistically significantly different from the control.

**Table 2:** Genes Down-Regulated with TSC + Hypoxia (each approximately a 40% decrease).

GENE	Hypoxia (Table 1)	Normoxia
GPX1	Up-regulated 227%*	Down-regulated 100% *
MYBL2	Up-regulated 199%*	Down-regulated 367%*
NOTCH 1	Up-regulated 253%*	No Change (0%)
CSTB	Up-regulated 209%*	Down-regulated 90%
SUMO 2	Up-regulated 205%*	Down-regulated 72%*

**Table 3:** Comparison of Hypoxia and Normoxia for Table 1 Genes.

GENE	Hypoxia (Table 2)	Normoxia
VEGF A	Down-regulated 43%*	No Change (0%)
B2M	Down-regulated 35%*	Up-regulated 26%*

**Table 4:** Comparison of Hypoxia and Normoxia for Table 2 Genes.

be seen, different changes in the expression of those genes occurred when the cells were grown under normoxia than when they were grown under hypoxia. Thus, it appears that the changes in the HIF-1 $\alpha$  gene expression with TSC are due to the modification of the hypoxic status of the cells rather than a direct effect on the genes themselves. As noted previously, TSC has not shown oxygen-enhancing effects in normal tissue in *in vivo* studies, so these results also support those observations.

It appears that TSC can be used to lessen the hypoxic state of malignant cells such as the U87 cell line. These results, combined with the previous studies in animal cancer models treated with both TSC and radiotherapy, strongly suggest that radiotherapy of cancerous tumors could be benefitted by the addition of TSC to that regimen. Since TSC has been found to be safe in humans [8], although for a different indication, it would appear to be a promising addition to the arsenal of anti-cancer options.

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