

Sulindac induces differentiation of glioblastoma stem cells making them more sensitive to oxidative stress

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Glioblastoma tumors (GBM) are very heterogeneous, being comprised of several cell subtypes, including glioblastoma stem cells (GSC). These tumors have a high rate of recurrence after initial treatment and one of the most prevalent theories to explain this is the cancer stem cell theory, which proposes that glioblastomas arise from mutations that transform normal neural stem cells (NSC) into GSC, which are highly resistant to oxidative stress and anti-cancer therapies. Sulindac is a non-steroidal anti-inflammatory drug (NSAID) that has been shown to protect the normal cells against oxidative damage by initiating a preconditioning response, but selectively sensitizes several cancer cell lines to agents that affect mitochondrial respiration, resulting in enhanced killing of the cancer cells. These effects of sulindac are independent of its NSAID activity. There is little information on the effect of sulindac on normal and cancer stem cells. To study the effect of sulindac on both normal and cancer stem cells, we have isolated normal neural stem cells (NSC) from mice hippocampi and glioblastoma stem cells (GSC) from a glioma cell line, U87.

As expected from previous studies, sulindac can protect normal astrocytes against oxidative stress. Sulindac induces differentiation of both NSC and GSC cells and sulindac upregulates neurogenesis in NSC. The differentiated NSC are also protected from oxidative stress damage, whereas the differentiation of GSC by sulindac increases the sensitivity of these cells to agents that cause oxidative stress. The S epimer of sulindac is more effective than the R epimer in inducing neuronal differentiation in both NSC and GSC. These results indicate that the ability of sulindac to induce GSC differentiation may have therapeutic value in preventing tumor recurrence.

Key words: glioblastoma, stem cells, sulindac, oxidative stress, differentiation

Neural stem cells (NSC) grow in the presence of growth factors such as EGF and/or bFGF and have the potential to differentiate into neurons, astrocytes, and oligodendrocytes [1]. NSC have been isolated and grown *in vitro* from several regions of the embryonic and adult central nervous system, such as the subventricular zone (SVZ) [2, 3], cortex [4], spinal cord [5, 6] and midbrain [7]. However, only two regions show *in vivo* neurogenesis in adults: the SVZ [8–11] and the subgranular layer of the dentate gyrus in the hippocampus [12–16]. The regulation of self-renewal and neurogenesis in the central nervous system (CNS) is in part dependent on the redox status within the cells. Elevated levels of reactive oxygen species (ROS) increase neurosphere production and neurogenesis, as well as glial differentiation [17–22]. As cancer stem cells share several characteristics with normal stem cells, it is reasonable to think that their generation could be the consequence of specific changes in embryonic

adult stem cells due to frequent cell divisions and mutation accumulation [23].

GBM are formed from a heterogeneous cell population. A subset of these cells retains the ability to repopulate the whole tumor when transplanted into mice [24]. This feature is the basis of the cancer stem cell theory, which states two important concepts: 1) tumors contain a number of cells that retain key stem cell properties and 2) tumorigenic cells arise from the transformation of tissue stem cells [25]. There is a clear relationship between the appearance of glioblastoma in NSC regions and its invasive and malignant features, supporting the theory that a specific transformation from a normal NSC to a glioblastoma stem cell (GSC) is involved in tumor initiation [26–28]. GSC are more resistant to traditional tumor treatments and they could be responsible for repopulating the heterogeneous GBM which would explain the high recurrence of tumors [25, 29–36]. Thus, GSC appear to be

an excellent target to prevent tumor reappearance. Effective therapies to avoid glioblastoma recurrence could include a) inhibiting the NSC to GSC transformation, b) specific killing of the GSC or c) making them more sensitive to anti-cancer treatment [37–40].

Sulindac is a non-steroidal anti-inflammatory drug (NSAID) that is known to have anti-cancer activity towards different types of cancer when used alone or in combination with other treatments [41–47]. In our previous studies, it was demonstrated that sulindac sensitized cancer cells to agents that perturb mitochondrial respiration resulting in enhanced death, and this effect was independent of its NSAID activity [48, 49]. In contrast, we found that sulindac could protect the normal heart and retinal pigmented epithelial (RPE) cells against oxidative damage by initiating an ischemic preconditioning response [50, 51]. These studies demonstrated the differential role of sulindac in protecting normal cells against oxidative damage, while enhancing the killing of several cancer cells under similar conditions.

As noted above, cancer stem cells are more resistant to chemotherapy than the cancer cells that are derived after differentiation. Based on earlier studies on the effect of sulindac on normal and cancer cells exposed to oxidative stress [48, 49] our initial goal was to extend these studies to both normal and cancer stem cells to see what effect sulindac might have when these cells were exposed to oxidative stress. For these studies we examined the effect of sulindac on normal astrocytes, NSC, a glioblastoma cell line (U87), and GSC, after exposure to oxidizing agents or anticancer drugs that affect mitochondrial respiration. Our studies support the protective role of sulindac on normal cells (both mature astrocytes and NSC) and its ability to enhance the sensitivity of glioblastoma cells to oxidative stress. An important new finding is that sulindac induces differentiation of both NSC and GSC, and that the GSC derived cancer cells show enhanced sensitivity to oxidative stress.

Materials and methods

Cell cultures. NSC were obtained from the hippocampi of P0 BL6 mice and cultured in DMEM/F12 medium (Gibco, Thermo Fisher Scientific, Waltham, MA) containing B27 (Gibco, Thermo Fisher Scientific, Waltham, MA), epidermal growth factor, EGF (Invitrogen, Thermo Fisher Scientific, Waltham, MA) and basic fibroblast growth factor, bFGF (Peprotech, Rocky Hill, NJ).

Neural stem cell differentiation and astrocyte isolation. To induce cell differentiation, NSC were plated on poly-L-lysine (PLL), obtained from Sigma, St. Louis, MO, where they spontaneously differentiate into neurons, astrocytes and oligodendrocytes as described elsewhere [52]. To obtain a pure culture of astrocytes, NSC were differentiated in DMEM + 10% FBS for seven days, trypsinized and re-plated in a new flask with the same medium. Under these circumstances, close to 100% of the cells become astrocytes (GFAP⁺ cells).

DMEM and FBS were obtained from Gibco, Thermo Fisher Scientific, Waltham, MA.

Isolation of glioblastoma stem cells. U87 were obtained from ATCC (ATCC, Manassas, VA). GSC were obtained from the U87 cell line following the protocol previously published [53]. Briefly, the U87 cells were cultured in the same culture medium as NSC. After a few days, floating neurospheres were formed. The floating GSC neurospheres were isolated, mechanically disassociated and grown in suspension following the same protocol used for NSC. After two passages, a pure culture of floating GSC neurospheres was obtained.

Treatments. Sulindac was obtained from Sigma (Sigma, St. Louis, MO). The R and S epimers of sulindac were obtained from Regis Technologies Inc, Morton Grove, IL. Two types of sulindac treatments were used for both NSC and GSC.

In treatment 1, we examined the effect of sulindac on floating, undifferentiated cells. In this treatment, the cells (both NSC and GSC) were treated as floating neurospheres for 24 hours with vehicle or sulindac and plated on PLL for another 24 hours in the presence of vehicle or drug, resulting in a total of 48 h of treatment.

The treatment 2 was used for GSC and NSC to observe the effect of sulindac on plated cells that were differentiated. After plating on poly-L-lysine (PLL), NSC start differentiating to neurons, astrocytes and oligodendrocytes. Cells were plated on PLL treated plates for five (GSC) or seven (NSC) days and treated for 48 hours with vehicle or sulindac. GSC were plated for 5 days, since these cells formed clusters and detached from the surface after 5 days of plating. Astrocytes (obtained as described in earlier section) were treated for 48 hours with vehicle or sulindac after 7 days post plating. U87 cells were treated for 48 hours, 24 hours after plating.

For GSC studies with dual drug combinations containing sulindac and anticancer drugs doxorubicin (DOX), dichloroacetate (DCA) and arsenic trioxide (As₂O₃), the drugs were co- incubated with sulindac and the GSC for 48 hours before viability was measured. When tert-butyl hydroperoxide (TBHP) was used, it was added for two hours after the GSC were incubated for 48 hours with sulindac, as described previously [48]. The anti-cancer drugs were obtained from Sigma. (Sigma, St. Louis, MO). Figure 1 summarizes the treatments described above that were used to differentiate both NSC and GSC.

Imaging and immunocytochemistry. Phase contrast pictures were obtained using an AmScope 10 MP camera attached to a Nikon TMS inverted microscope. Indirect immunocytochemistry (ICC) was performed as described previously [52, 54, 55]. Briefly, NSC were mechanically disaggregated and plated on PLL. For ICC, the following markers were used: monoclonal anti-nestin (clone Rat 401; 1:200, obtained from Developmental Studies Hybridoma Bank. University of Iowa, Iowa City, IA) for NSC staining and polyclonal anti- β -tubulin isotype III (1:2000; Covance, BioLegend, San Diego, CA 92121) for neuronal identifica-

tion. Epifluorescence microscopes (Leica and Nikon) were used for pictures, counting and visualization of the immunocytochemistry. The total number of neurons was quantified by counting a minimum of 15 fields per treatment in triplicates or quadruplicates. The number of positive neurons was corrected for total cells in the same area, quantified with nuclear staining with Hoechst (Sigma-Aldrich, St. Louis, MO 63103), and normalized to non-treated cultures.

Western blot. Protein extraction for western blot and enzyme activity analysis was performed as described elsewhere [55]. We also used nestin, β -tubulin III and DCX (a marker for neuronal progenitor cells) and actin (Cell signaling Technologies, Danvers, MA) as a loading control for the detection of the different cell types by western blot.

Cell viability assay. NSC, GSC, astrocytes and U87 cells were plated at 10,000 cells per well in a PLL coated 96-well plate and the cell viability was measured as previously published [49]. Briefly, the cells were grown at 37°C in a 5% CO₂ incubator for a specified time, the medium discarded under aseptic conditions and replaced with fresh culture medium containing the indicated drug combinations for specified times described in the Results section. The culture medium was discarded and the cells were washed with PBS. Cell viability was determined by using the CellTiter 96 Aqueous One Cell Proliferation Assay (Promega, Madison, WI) according to the manufacturer's instructions. Cell viability was quantified by measuring the absorbance at 490 nm using a colorimetric microtiter plate reader (SpectraMax Plus; Molecular Devices). Background absorbance was subtracted from each sample. The graphs represent the percentage of cell survival compared to the control without TBHP treatment.

Statistical analysis. Analysis of variance (ANOVA) and multicomparison post hoc test (Bonferroni), Student's t-test and additional statistics were performed using the Prism4

program from GraphPad Software Inc. The graphs are represented as SE \pm mean. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Results

Isolation of NSC and GSC. As previously reported, NSC can grow almost indefinitely in culture in floating clusters of dividing cells called neurospheres (Figure 1, Figure 2A) [1, 7, 56, 57]. NSC stop proliferating and start differentiating after attaching to an adherent substrate (PLL). Under these circumstances, NSC differentiate into neurons, astrocytes and hardly detectable levels of oligodendrocytes, plus a relatively small percentage of glial and neuronal progenitor cells, as previously published (Figure 2B) [57]. We isolated pure astrocytes from NSC by maintaining the NSC culture in 10% FBS for several passages, as described in methods.

To isolate GSC, we used the U87 glioblastoma cancer cell line as described previously [53]. U87 cells (Figure 2C) when cultured in the presence of EGF and bFGF, yield GSC that grow as floating clusters of cells, similarly to NSC neurospheres (Figure 2D).

Sulindac induces differentiation of NSC and GSC. Under the various growth conditions summarized in Figure 1, it is possible to compare the effect of sulindac on (i) normal cells (astrocytes and NSC) vs cancer cells (U87 and GSC) and (ii) stem cells before and after differentiation.

Figure 3 shows the increase in neuronal differentiation obtained after treating NSC with sulindac in both treatments 1 and 2. Figure 3A shows that NSC 24 hours post plating show a relatively low number of neurons (β -tubulin III⁺ cells, red) without any drug. However, the number of neurons increased significantly after sulindac treatment (Figure 3B). Similar differences were found after five days post plating as seen with control NSC in Figure 3C compared to cells treated

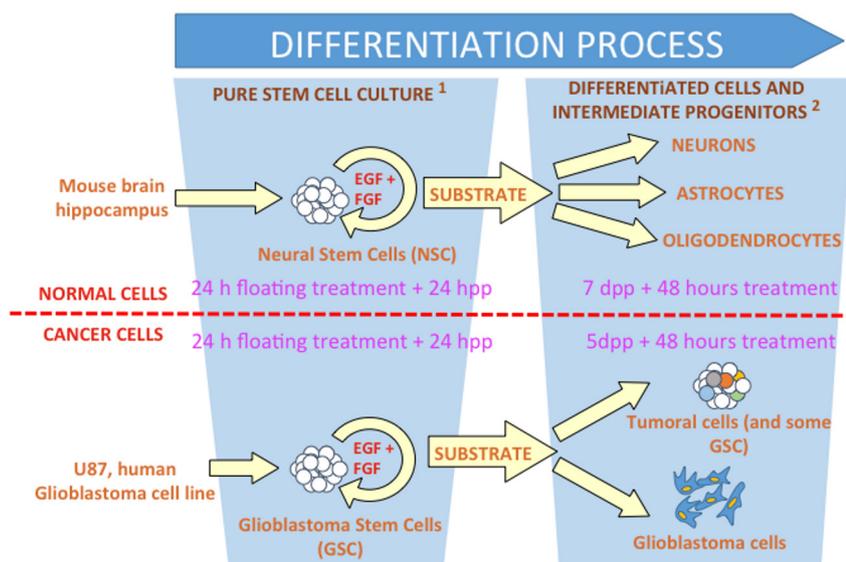


Figure 1. Treatments used to differentiate both normal neural and glioblastoma stem cells.

¹UNDIFFERENTIATED CELLS: Floating NSC contain mostly nestin⁺ undifferentiated cells. In order to see sulindac effect on undifferentiated stem cells, floating neurospheres (for both NSC and GSC) were treated for 24 hours with sulindac. Cells were then plated on PLL and quantified 24 hours post-plating (hpp).

²DIFFERENTIATED CELLS: 7 days post-plating (dpp), NSC progeny differentiates to mature neurons and glial cells with some glial and neuronal progenitors and almost no undifferentiated stem cells are present in the culture. NSC progeny was then treated for 48 hours with sulindac. Similarly, GSC were plated for 5 days before being treated with sulindac for 48 hours, to have a comparison normal vs. cancer cells.

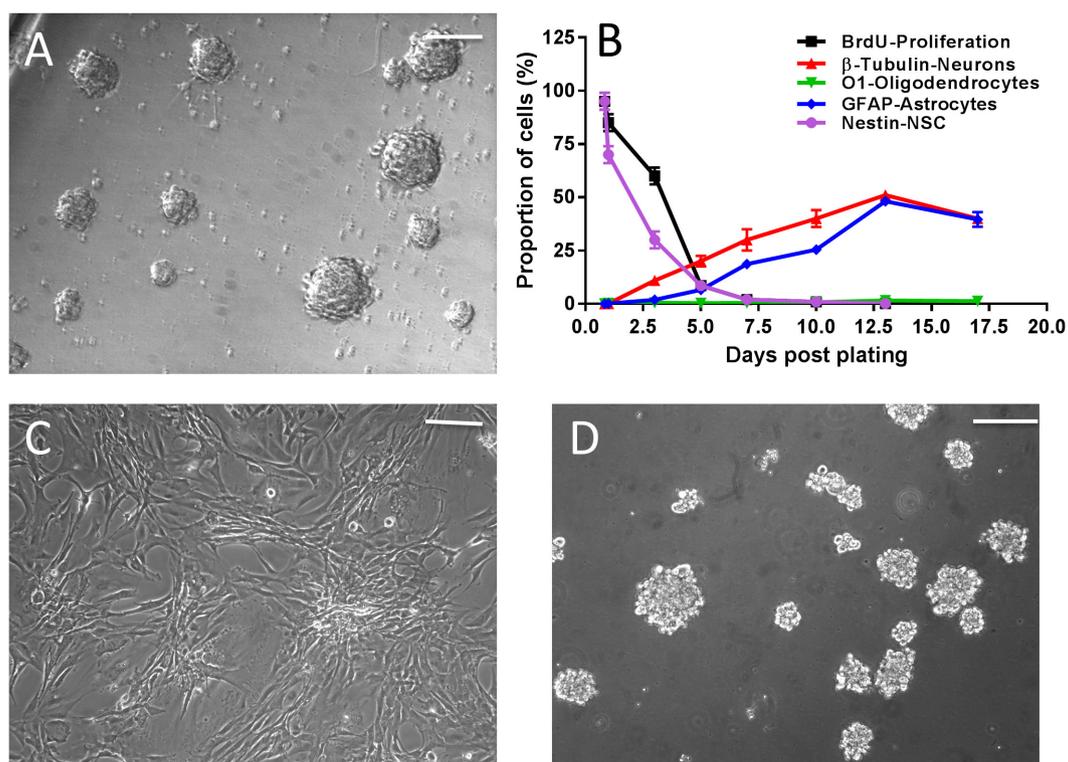


Figure 2. Cell culture of neural stem cells and isolation of glioblastoma stem cells. (A) Neural stem cells (NSC) grown in culture in the presence of EGF and bFGF as undifferentiated floating clusters of cells called neurospheres, (B) Time course spontaneous differentiation of NSC toward neurons, astrocytes and oligodendrocytes after plating them on poly-L-lysine. Adapted from [57], (C) U87 glioblastoma cell line, (D) Floating glioblastoma stem cells (GSC) derived from U87 cells growing as neurospheres in the presence of EGF and bFGF.

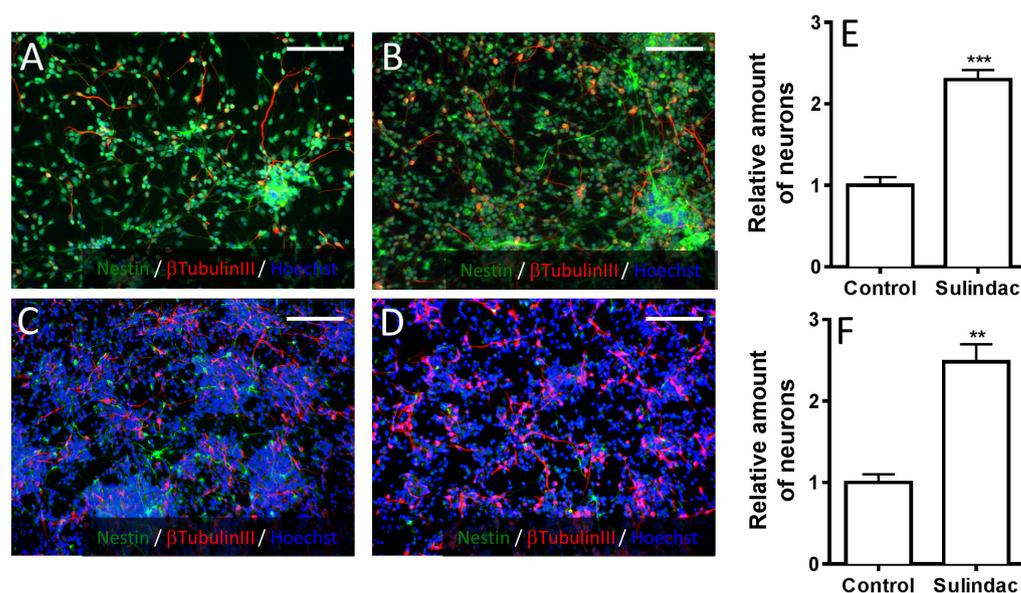


Figure 3. Sulindac induces neuronal differentiation of NSC. Photomicrographs with the neuronal-specific antibody β -tubulin III (red) and NSC-specific antibody nestin (green) performed at different time points, counterstained with the nuclear stain Hoechst (blue). (A) NSC treated with vehicle for 24 h and then plated on PLL for 24 h, (B) NSC treated with sulindac for 24 h and then plated on PLL for 24 h, (C) NSC plated on PLL for 7 days and treated with vehicle for 48 h, (D) NSC plated on PLL for 7 days and treated with sulindac for 48 h, (E) Comparison of percentage of neurons in NSC control and sulindac treated NSC 7 days after plating and treatment with vehicle/sulindac for 48 h. Sulindac concentration used: 500 μ M.

with sulindac (Figure 3D). The bar graphs in Figures 3E and Figure 3F show the percentage of neurons, untreated vs treated after 24 hours and 7 days post plating.

Similar to NSC, floating GSC were mechanically dissociated and forced to attach to PLL. After plating, a high percentage of GSC remained growing as attached clusters both at 24 hours and 5 days (Figures 4 A, C). However, sulindac addition to cells both 24 hours post plating

(Figure 4B) and 5 days post plating (Figure 4D) shows a clear differentiation effect, with most cells attached to the plate forming a monolayer.

To test whether the morphological differentiation observed in Figures 4B and 4D after sulindac treatment corresponds to changes in the cellular phenotype, a series of experiments were performed to measure the levels of several cell-specific protein markers by western blot (Figure 4E).

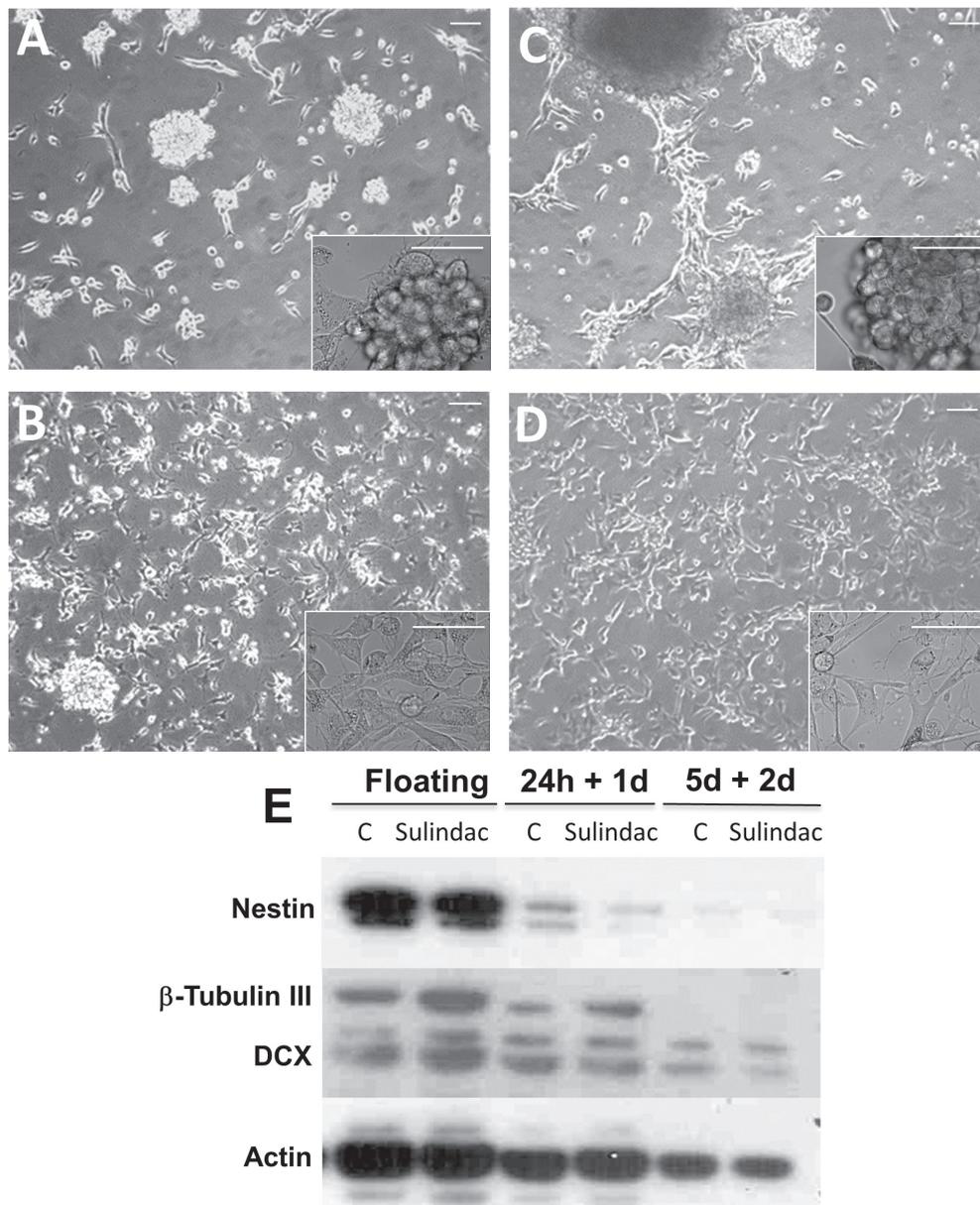


Figure 4. Sulindac induces morphological cell differentiation and increases neuronal markers in GSC. Low magnification phase contrast view of GSC with inserts showing higher magnification. Floating cells subjected to 24 h treatment followed by one day (24 hours) plating with (A) vehicle and (B) sulindac. GSC plated for 5 days followed by 48 h treatment with (C) vehicle and (D) sulindac. (E) Representative western blots: Floating: GSC treated in suspension for 24 hours, 24 h + 1d: GSC treated in suspension for 24 (Floating), followed by the same treatment for another 24 hours postplating and 5d + 2d: GSC treated for 2 days at the 5th dpp. The samples were probed with antibodies against nestin, β -Tubulin III and doublecortin (DCX). Sulindac concentration used: 500 μ M. Bar: 50 microns.

Beta-tubulin III, a microtubule protein specific for neurons and doublecortin (DCX), a microtubule-associated protein expressed in neuronal precursor cells, were used to evaluate neuronal differentiation. As seen in Figure 4E, sulindac decreases the nestin levels and appears to induce neuronal differentiation of GSC with all treatments. Beta-tubulin III levels are low in control cells but when treated with sulindac, there is an increased expression in floating and 24 h post plating of differentiation while that of DCX did not

change significantly. 5 days after plating, the expression of both Nestin and Beta-tubulin III markers decreased almost until undetectable levels were observed.

Sulindac differentially affects cell survival in NSC, GSC, astrocytes and glioblastoma cells exposed to oxidative stress. Our previous studies have shown a dual effect of sulindac since it can enhance the killing of cancer cells against oxidative stress while protecting normal cells under similar conditions [48, 49] In order to determine the sensi-

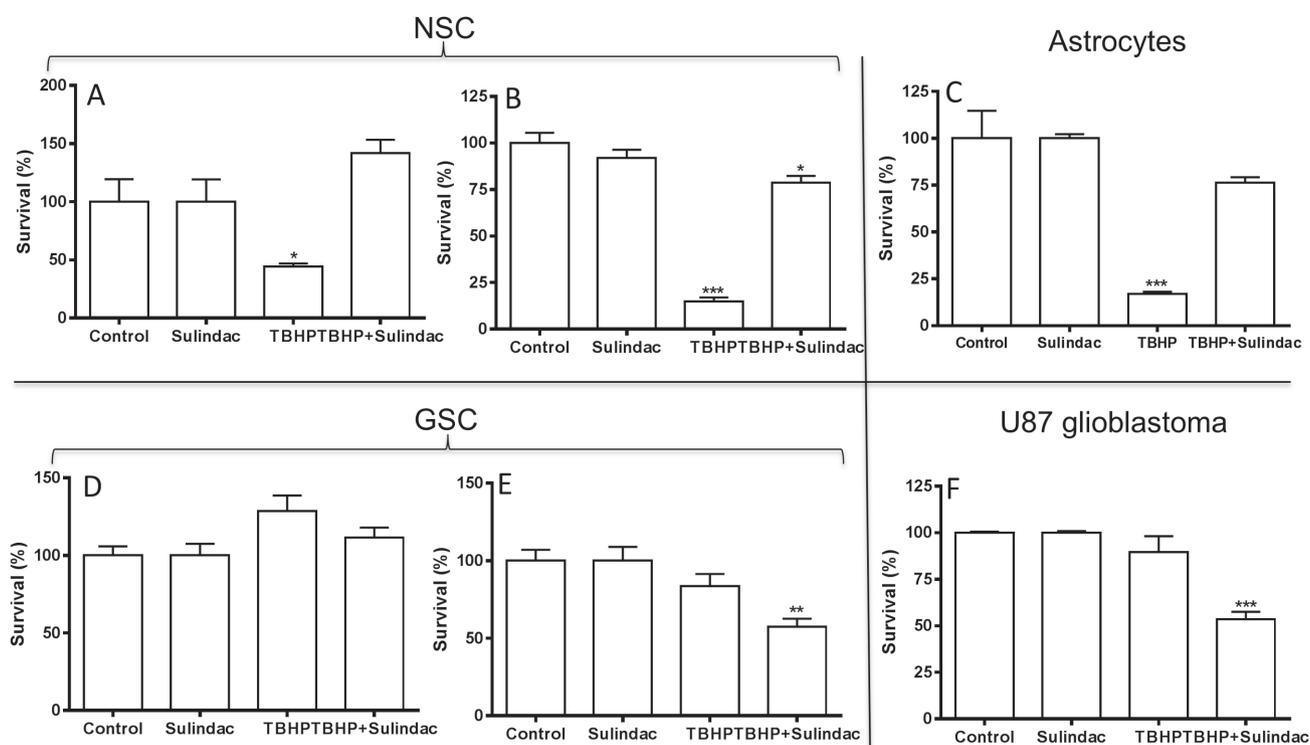


Figure 5. Sulindac differentially affects cell survival in NSC, astrocytes, U87 and GSC exposed to TBHP oxidative damage. Cell viability was determined using MTS assay (see methods). Effect of sulindac on (A) NSC after 24 h treatment, (B) NSC plated for seven days followed by 48 h treatment, (C) astrocytes plated for seven days followed by 48 h treatment, (D) GSC as floating neurospheres followed by treatment for 24 hours, (E) GSC plated for five days followed by 48 h treatment, (F) U87 plated for 24 h followed by 48 h treatment. Sulindac concentration was 500 μ M and the TBHP concentration was 200 μ M.

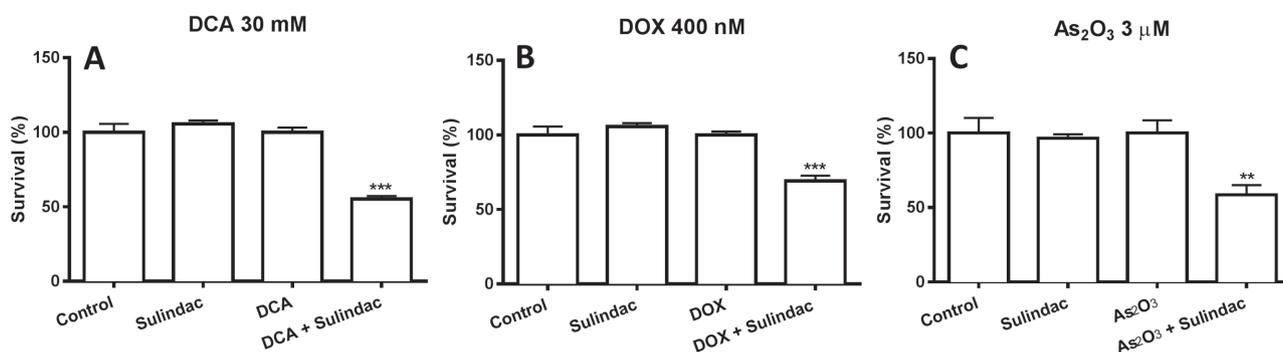


Figure 6. Sulindac sensitizes GSC to anticancer and oxidizing agents. Cell viability was determined in GSC plated for five days and treated for two days with vehicle or 500 μ M sulindac in the presence or absence of DCA, 30 mM, DOX, 400 nM and As₂O₃, 3 μ M. (A) sulindac and DCA, (B) sulindac and DOX (C) sulindac and As₂O₃.

tivity of astrocytes, U87 glioblastoma cells, GSC and NSC to oxidative stress, tert-butyl hydroperoxide (TBHP) was used as the oxidizing agent, in the presence or absence of sulindac. The results are presented in Figure 5. After 24 h treatment, sulindac protects floating NSC from TBHP-induced death as seen in Figure 5A. After 7 days, sulindac also almost completely protects the NSC against TBHP-induced cell death (Figure 5B). A very similar protection with sulindac is also observed in cultured astrocytes treated with TBHP (Figure 5C). However, floating undifferentiated GSC were very resistant to oxidative stress, and show no enhanced killing when treated for 24 hours with sulindac (Figure 5D). In contrast, when GSC cells were plated for five days and then treated for two days with sulindac, the cells showed higher sensitivity to TBHP and sulindac treatment resulted in enhanced killing (Figure 5E), as compared with control NSC cells. A similar enhanced killing effect is observed in U87 glioblastoma cells treated with sulindac for 48 hours (Figure 5F).

These results suggest a protective effect of sulindac against oxidative stress in NSC and astrocytes with little or no effect on floating GSC. In contrast, sulindac increases sensitivity of U87 cells and differentiated GSC to oxidative stress, as shown previously with other cancer cell lines [48, 49]. In summary, these results support previous stem cell results, since GSC, like other stem cells, are very resistant to oxidative stress, but upon differentiation they behave like U87 cells.

Sulindac sensitizes differentiated GSC to agents that induce oxidative stress. In order to study the effect of

sulindac in enhancing GSC killing after treatment with known anti-cancer drugs, the GSC cells were treated for 5 days, followed by a 2 day treatment with sulindac and/or the anticancer drugs, dichloroacetic acid (DCA), doxorubicin (DOX), or As_2O_3 (Figure 6 A–C). Dosage of these drugs was chosen based on earlier studies ([49, 58–60]). These anticancer drugs are known to cause oxidative stress in the cancer cells. As shown in Figure 6, under the conditions used, sulindac alone or the anticancer drugs by themselves showed little killing. However, there is enhanced killing of the differentiated GSC when sulindac is used in combination with any of the three different anticancer agents.

Sulindac-induced differentiation is independent of its NSAID activity. In order to see if the mechanism involved in the sulindac-induced cell differentiation of GSC is due to NSAID activity, ibuprofen, another NSAID was tested as well as sulindac sulphone, a sulindac metabolite that has no NSAID activity. Also, sulindac has a chiral sulfur center and it is an equal mixture of the R and S epimers and the individual epimers were also tested [61]. The results are shown in Figure 7. Compared to the control cells (Figure 7A), sulindac (an equal mixture of the R and S epimers) shows significant differentiation (Figure 7B). Sulindac sulfone also shows weak differentiation activity (Figure 7C), whereas ibuprofen has no activity (Figure 7D). The sulindac R-epimer is also inactive (Figure 7E), but the sulindac S epimer is the most potent differentiation agent (Figure 7F). It should be noted that sulindac sulfone was used at a much lower concentration because of its toxicity. These results indicate that the

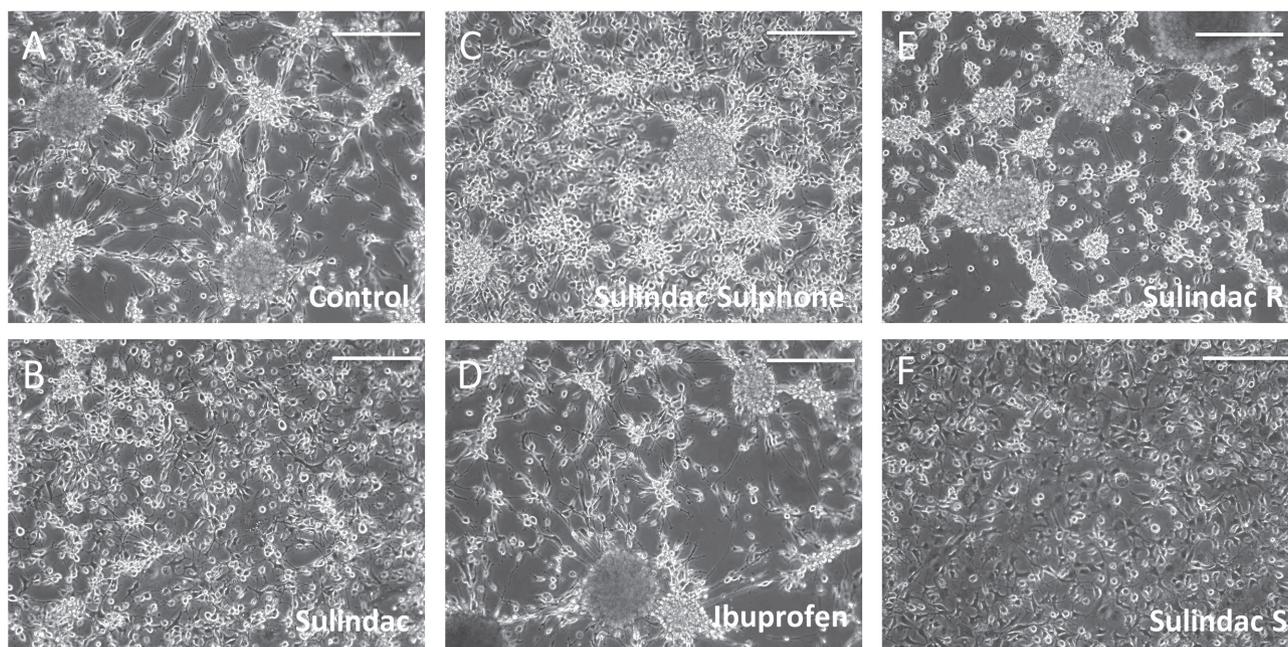


Figure 7. Sulindac-induced differentiation of GSC is independent of its NSAID activity and specific for its S epimer. Following 5-day post plating, GSC were treated for 48 h with (A) vehicle, (B) 500 μ M sulindac, (C) 25 μ M of sulindac sulphone, (D) 400 μ M of ibuprofen, (E) 250 μ M of the R epimer of sulindac or (F) 250 μ M of the S epimer of sulindac.

sulindac differentiating effect is independent of its NSAID activity, and appears to be primarily due to the S epimer of sulindac.

Our results confirm and extend our previous findings that sulindac protects normal cells against oxidative stress, but enhances the killing of cancer cells when exposed to oxidative stress. In addition, sulindac stimulates cell differentiation of both NSC and GSC and more importantly, makes GSC more sensitive to oxidative stress.

Discussion

A major challenge in cancer therapy is to develop drugs that kill tumor cells without being toxic to normal cells and avoid cancer relapse by eliminating all possible cancer and cancer stem cells. Cancer stem cells are very resistant to chemotherapeutic agents and retain their ability to repopulate the whole heterogeneous tumor cell population [62]. There have been several studies focusing on differentiating cancer stem cells (CSC) of different tissues into a more differentiated state. All-trans retinoic acid has been successfully used in patients suffering from acute promyelocytic leukaemia, with or without combination with other chemotherapy agents [63, 64]. Phorbol myristate acetate [65, 66] and dimethylsulfoxide [67] also induced maturation of the leukemic cell lines. A more effective treatment based on stimulation of CSC differentiation therapy would use a dual drug combination including a differentiating agent (s) with an anticancer drug(s), in order to eradicate both the CSC and non-CSC. One reported example involves inhibition of TGF beta, which induces the differentiation of breast CSC, in combination with paclitaxel to eliminate the fast proliferating non-CSC forming in the tumor [68]. Prabhakaran et al. [69] have shown that cisplatin inhibits breast cancer cell survival and also induces differentiation of breast cancer stem cells. There are other treatments able to induce cell differentiation. It has been reported that some growth factors have differentiating effects on normal brain cells, like BDNF and NGF. However, these growth factors have possible tumorigenic effects [70–72]. Our previous results showed that sulindac, in combination with DCA enhances cancer cell killing in several types of cancer *in vitro* [49].

In the case of glioblastoma, the heterogeneity of the tumor and the role of the stem cell microenvironment (niche) in the cancer progression has been studied [73]. It has been shown that GSC possess elevated resistance to chemotherapy and have a high capacity to regenerate the tumor by differentiation into highly proliferative cells [74]. There have been several approaches for eliminating this kind of tumor by targeting GSC [75]. The present study provides evidence that sulindac induces differentiation of GSC, making them susceptible to anti-cancer treatments. Sulindac is unique in its differentiation ability in comparison to other agents, since it can sensitize glioblastoma cells to oxidative damage while protecting normal glial cells against oxidative damage. The

present results show that sulindac induces cell differentiation of both NSC and GSC and transforms GSC to a more differentiated cell type. Following treatment with sulindac, there was a decrease in nestin immunopositivity in both NSC and GSC indicative of loss of stem-like characteristics. In case of NSC, sulindac significantly augmented the beta tubulin expression pushing NSC towards neuronal phenotype. Sulindac treatment increased beta tubulin expression in GSC at specific time points. However, it was more complicated in GSC since there was an overall decrease in beta tubulin after few days in culture. It has been reported that GSC isolated from established cultures show significant decrease in beta tubulin expression after many days in culture [76]. This is also in agreement with other studies that show loss of beta tubulin expression in GSC cells under differentiation conditions [77]. Our results, consistent with previous literature suggest that GSC tend to differentiate more towards their original parental cell lineage [76, 77].

We have shown that sulindac-induced differentiation made GSC more susceptible to TBHP or drugs used in anti-cancer therapy, like DOX, DCA or As_2O_3 that create oxidative stress in the cancer cells. To our knowledge, there is only one other report indicating that sulindac can differentiate stem cells. Singh et al. showed that sulindac induced differentiation markers such as CD14, CD15 and CD115 in a population of human AML cells [78]. There is a clinical relevance to understanding this effect of sulindac since there are recent reports that suggest that forcing GSC to differentiate into neurons or astrocytes is one of the effective methods to reduce tumor growth or recurrence [79]. This morphological differentiation and decreased cell proliferation seems to be related to higher sensitivity of these differentiated cells to oxidative stress.

The mechanism by which sulindac induces neuronal differentiation is not clear. However, several authors have previously described a variety of mechanisms involved in glioblastoma proliferation and malignancy [80]. Supporting the hypothesis of common mechanisms of self-renewal and differentiation in the context of the cancer stem cell theory [81], our results show similarities between NSC and GSC in regards to their differentiation response to sulindac [82, 83]. The morphological changes observed, with no clusters of proliferating cells, suggest that both sulindac and specifically the S epimer induce cell differentiation and the exit of the cells out of the cell cycle. That, by itself would be a positive step in stopping cancer progression. Although the R epimer of sulindac did not appear to induce morphological differentiation of GSC, the cells did have a phenotypic change since they also became more sensitive to anti-cancer drugs. The effect of the R epimer may be a way to identify metabolic changes related to oxidative stress that occur before definitive signs of differentiation are observed.

It is known that in NSC redox balance plays a very important role in their maintenance and proliferation, as well as in their neuronal and glial differentiation [84, 85]. Antioxidants like edaravone promote proliferation of NSC [86] and

COX-2 regulates the proliferation of cells that have glioma stem like properties [87]. There have been other reports of the effects of non-steroidal anti-inflammatory drugs (NSAIDs) on proliferation and differentiation of NSC [87], as well as on CSC [88]. Sulindac is a prodrug with NSAID properties, and previous studies have shown that MsrA can reduce the S epimer of sulindac to sulindac sulfide, an active COX inhibitor, and MsrB, or an MsrB like activity present in liver can reduce the R epimer to the active NSAID [61]. However, as shown in Figure 7, ibuprofen, a known NSAID, was unable to induce the GSC differentiation. In addition, sulindac sulfone, the oxidized metabolite of sulindac, which has no NSAID activity, could partially induce cell differentiation. Finally, the two epimers of sulindac have different abilities to differentiate stem cells, although both are NSAID prodrugs. These results suggest that the major differentiating effect of sulindac is not due to its ability to inhibit the COX enzymes. Our earlier studies showed that sulindac functioned as an ischemic preconditioning agent (IPC) in protecting the heart and retinal pigmented epithelial cells against oxidative damage resulting from ischemia/reperfusion treatment and from chemical oxidation [50, 51]. There is no evidence yet that a preconditioning response is involved in the sulindac induced differentiation of both NSC and GSC.

The sulindac-induced enhanced killing of cancer cells exposed to oxidative stress has been previously described [48, 49], but the mechanism by which sulindac sensitizes cancer cells to agents that affect mitochondrial function is also not fully understood. The previous studies indicated that this effect of sulindac may be related to a fundamental metabolic difference between how normal and cancer cells generate energy through respiration, as first described by Warburg [89]. Cancer cells appear to have a defect in their respiratory chain and obtain as much as 40% of their energy from glycolysis even in the presence of adequate oxygen, whereas glycolysis in normal cells accounts for less than 5% of the energy requirement. Many studies have shown that cancer cells are more sensitive to glycolysis inhibitors than normal cells [90, 91] and other studies have provided evidence that compounds that affect the mitochondria are more toxic to cancer cells than normal cells [92]. Our previous work showed that sulindac enhances cancer killing when used in combination with agents that affect mitochondrial function [49]. What is clear is that reactive oxygen species (ROS) are involved in both the preconditioning protective response elicited by sulindac in normal cells and sulindac's ability to enhance the killing of cancer cells exposed to oxidative stress, indicating a major role of mitochondria in both effects [50, 51].

Although much of our previous work has been *in vitro*, there are two clinical studies that support our cell culture results on cancer killing. A previous limited proof of concept clinical study demonstrated that sulindac, in combination with hydrogen peroxide, can be used as a treatment for precancerous skin squamous cells (actinic keratosis) [93]. In a much larger clinical study on the recurrence of advanced

colon polyps, it was shown that the combination of sulindac and difluormethylornithine (DFMO) reduced the recurrence of polyps and the appearance of adenocarcinoma in these patients by >80% over a 3 year period [47, 94].

The use of sulindac as a differentiating agent for GSC in combination with chemotherapy has some advantages compared with other known compounds. Sulindac is inexpensive, it has low toxicity and has been used clinically as an NSAID for many years. There is also evidence that it can cross the blood brain barrier. [61]. In addition, sulindac enhances cancer cell killing in the presence of oxidative stress [48, 49] and exerts a protective effect on normal cells against oxidative damage [50, 51].

Conclusion

Glioblastoma is one of the most aggressive types of cancer that appears to resist all types of treatment approaches. In the heterogenous population of glioblastoma tumor cells, the stem cells confer the most resistance to therapies. In the present studies we demonstrate that sulindac induces GSC to shift to a more differentiated cell type and in addition, sulindac enhances selective tumor cell killing in the presence of both oxidizing and anti-cancer agents. Further studies are needed to understand the mechanism of action of sulindac in differentiating and selective sensitizing of cancer cells.

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