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# Salinomycin's potential to eliminate glioblastoma stem cells and treat glioblastoma multiforme (Review)

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**Abstract.** Glioblastoma multiforme (GBM) is the most common and deadliest form of primary brain tumor. Despite treatment with surgery, radiotherapy, and chemotherapy with the drug temozolomide, the expected survival after diagnosis remains low. The median survival is only 14.6 months and the two-year survival is a mere 30%. One reason for this is the heterogeneity of GBM including the presence of glioblastoma cancer stem cells (GSCs). GSCs are a subset of cells with the unique ability to proliferate, differentiate, and create tumors. GSCs are resistant to chemotherapy and radiation and thought to play an important role in recurrence. In order to effectively treat GBM, a drug must be identified that can kill GSCs. The ionophore salinomycin has been shown to kill cancer stem cells and is therefore a promising future treatment for GBM. This study focuses on salinomycin's potential to treat GBM including its ability to reduce the CSC population, its toxicity to normal brain cells, its mechanism of action, and its potential for combination treatment.

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## 1. Introduction

Glioblastoma multiforme (GBM) is the most common and deadliest form of primary brain tumor. While current standard treatment includes a rigorous regimen of surgery, radiotherapy, and chemotherapy with temozolomide, its effectiveness remains low. The median survival rate for the condition is only 14.6 months and the two-year survival rate is a mere 30% (1,2). Temozolomide is the only FDA-approved chemotherapy for primary GBM, highlighting the need for better drugs to improve patient outcomes. One possible reason for this observed treatment resistance is the heterogeneity of GBM. Studies have shown that many types of cancers including GBM contain a subpopulation of cells known as cancer stem cells (CSCs) (3-6). CSCs are the only cells that have the ability to proliferate, differentiate, and generate tumors. Because of this, they are important to recurrence and metastasis. CSCs have also been found resistant to radiation and chemotherapy (7,8). Investigations on the susceptibility of glioblastoma cancer stem cells (GSCs) to temozolomide have conflicting results, with some finding depletion and others, enrichment (9-13). These studies have also found a variety of genetic factors that prevent temozolomide effectiveness on GBM including the methylation status of the O(6)-methylguanine-DNA-methyltransferase (MGMT) promoter, p53 mutations, and ATP-binding cassette (ABC) transporters. In order to effectively treat glioblastoma, a drug must be identified that can target GSCs and overcome these mechanisms of resistance.

One emerging drug for targeting GSCs is salinomycin. Salinomycin is an ionophore with a preference for $Na^+$ and $K^+$ that was isolated from *Streptomyces albus* (14). Salinomycin has antimicrobial activity and has been used since the 1980s as a coccidiostat and growth promoter (15-18). It was not until a 2009 study by Gupta *et al* that salinomycin's selectivity for CSCs was elucidated (19). In this study, 16,000 compounds were screened for their ability to kill breast cancer stem-like cells created by the knockout of CDH1 with shRNA. This cell population displayed all of the primary CSC characteristics including tumorsphere formation, chemoresistance, CSC marker expression, and tumor seeding ability. Salinomycin was found to kill these cells with a greater than 10-fold lower $IC_{50}$ as compared to the non-knockout population and reduce tumor seeding ability in mice by 100-fold. Since this initial identification of salinomycin as having CSC potency, researchers have discovered effectiveness against numerous other types of cancer stem cells including chronic lymphocytic leukemia, prostate cancer, colorectal cancer, and lung adenocarcinoma (20-24). Furthermore, unlike temozolomide, salinomycin has been shown to act independently of p53 and

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is able to overcome ABC transporters (25,26). Salinomycin's mechanism of action however remains to be clearly identified. While some researchers have found evidence of apoptosis, others have indicated autophagy, and still others have identified the mechanism as controlled necrosis (25-28). This study focuses on salinomycin's potential to treat GBM including its ability to reduce the CSC population, its toxicity to normal brain cells, its mechanism of action, and its potential for combination treatment.

## 2. Salinomycin's effect on GSCs

There are a variety of methods to test the effect of salinomycin on GSCs. One method is comparing the potency of salinomycin on cells that are enriched for GSCs to those that are not. In 2015, Chen *et al* grew the GL261 GBM line in both GSC-enriching neurosphere culture and differentiation-inducing adherent culture (29). They found the neurosphere culture cells to be much more sensitive to salinomycin than the adherent culture cells, suggesting an increased toxicity in GSCs. Using similar logic, Xipell *et al* determined the viability of 18 different cell lines when treated with salinomycin (30). They compared the $IC_{50}$ values of the neurosphere cultures and adherent cultures and found the neurosphere cultures had significantly lower $IC_{50}$ dosages.

Another method to measure stemness is by determining the ability of cells to form neurospheres *in vitro*. This is known as a clonogenicity assay and has been conducted on salinomycin treated GBM in studies by Chen *et al* in 2015, Qin *et al* in 2015, and Xipell *et al* in 2016 (27,29,30). The ability to form neurospheres is a mark of stemness, so inhibiting this ability is evidence of a drug that targets GSCs. Chen *et al* and Qin *et al* both demonstrated salinomycin's ability to decrease clonogenicity (27,29). Furthermore, Xipell *et al* tested multiple salinomycin concentrations finding a dose-dependent decrease in neurosphere formation (30).

A final criterion that has been employed to test the effect of salinomycin on GSCs is gene expression. A variety of markers have been used to identify GSCs including CD133, SOX2, Nestin, and Musashi-1 (5,31-33). However, only one study has investigated the effect of salinomycin on these GSC markers. Xipell *et al* used qRT-PCR to show that an unspecified concentration of salinomycin resulted in decreased express of the GSC markers Musashi, Sox2, and Nestin in one cell line (30).

Together these three indicators provide evidence of salinomycin's ability to kill GSCs. In order to confirm this hypothesis though, more data are needed. GSC marker gene expression should be repeated with additional cell lines. The effect of salinomycin on GSC markers should also be assessed using alternative methods such as flow cytometry and western blotting. Most importantly, salinomycin's ability to reduce GBM tumor seeding ability in mice should be assessed as it was for breast cancer in Gupta *et al* (19).

## 3. Normal neural cell toxicity

A potentially significant limitation to the use of salinomycin for killing GSCs is its toxicity to normal neural cells. An outbreak of toxic polyneuropathy in cats occurred in the Netherlands in 1996 as a result of cat food contaminated with salinomycin at a level of 13-21 ppm (34). Of the estimated 100,000 exposed cats, 823 developed acute paralysis which began in the hindlimbs and then, for some cats, progressed to the forelimbs. Morphological findings included loss of axons and Schwann cell swelling. Salinomycin toxicity has also been identified in dogs, horses, and even humans (35-37). In 2004, a 35-year-old man working in a factory making animal feed accidentally inhaled/ingested an estimated 1 mg/kg body weight of salinomycin. He developed nausea, shortness of breath, and dizziness within minutes and complained of leg weakness once arriving at the hospital. The patient subsequently developed rhabdomyolysis and was not able to be discharged until 40 days after exposure (37).

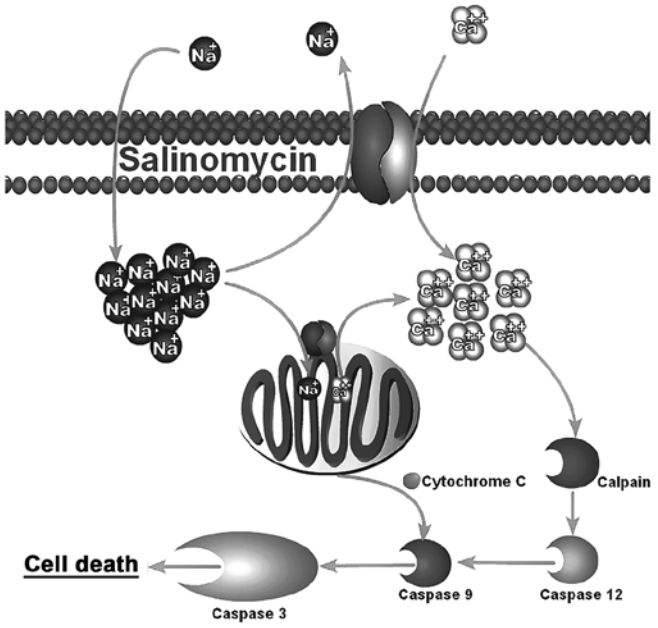


Figure 1. Summary of salinomycin's mechanism of action on neurons. Salinomycin transports $Na^+$ into the cell, causing the plasma membrane and mitochondrial NCXs to work in reverse leading to an increase in cytosolic $Ca^{2+}$. Elevated intracellular $Ca^{2+}$ levels activate calpain, which in turn activates the caspase pathway. Cytochrome *c* released from the depolarized mitochondria also activates caspase-9. Caspase-activation results in cell death via apoptosis (38). Reprinted with permission from Nature (72).

In 2011, Boehmerle *et al* investigated this neural cell toxicity using dorsal root ganglia neurons and Schwann cells from mice (38). They found significant viability reductions in both cell types when treated with salinomycin. They discovered this death was occurring via apoptosis and is a result of salinomycin's action as a sodium ionophore. Salinomycin causes an upregulation of intracellular sodium which subsequently causes an intracellular calcium upregulation due to reversal of the $Na^+/Ca^{2+}$ exchangers (39). This intracellular calcium influx then leads to calpain and cytochrome *c*-mediated apoptosis (38). This mechanism is shown in Fig. 1.

In order for salinomycin to be utilized as a clinically effective treatment against GBM, salinomycin must either 1) be more sensitive to calcium induced apoptosis than normal cells or 2) act through a different mechanism that is specific to cancer cells. In the former case, a dosage high enough to kill GSCs but low enough to prevent neurotoxicity could be

Table I. Evidence for the three different cell death pathways.

| Cell death pathways | Evidence | Number of studies | | Refs. |
|---|---|---|---|---|
| | | Yes | No | |
| Apoptosis | Annexin V/PI Increase | 2 | 2 | (27,29,30,62) |
| | Caspase-3 cleavage | 2 | 3 | (27,29,30,62,63) |
| | Increased caspase-3 activity | 1 | 1 | (27,30) |
| Autophagy | p62 accumulation | 2 | 0 | (30,63) |
| | LC3-II upregulation | 2 | 0 | (30,63) |
| Programmed necrosis | ROS increase | 3 | 0 | (27,30,63) |
| | Mitochondrial PTP opening | 3 | 0 | (27,30,62) |

used to selectively kill GSCs without harming normal cells. This potential selectivity may be due to the presence of a greater concentration of $Na^+/Ca^{2+}$ exchangers or a higher than normal intracellular calcium concentration in GSCs. Calcium is a secondary messenger in the Wnt pathway which has been found to be expressed in GBM, providing a possible explanation (40,41). If this is not the case, salinomycin may act through a different, GSC-specific mechanism. Under this scenario, salinomycin could be used as an effective treatment whether or not the salinomycin concentration required to kill GSCs is less than the concentration which kills normal cells. In the case where the required salinomycin dose is low, salinomycin alone would be effective in killing GSCs without causing normal cell toxicity. However, if the salinomycin dose required to eliminate GSCs is similar to that causing normal cell toxicity, salinomycin could be administered along with a $Na^+/Ca^{2+}$ exchanger inhibitor. This inhibitor would prevent salinomycin induced apoptosis of normal neural cells while allowing salinomycin to kill GSC through GSC-specific mechanisms. Therefore, understanding salinomycin's mechanism of action is of great importance to understanding salinomycin's safety and potential for clinical application.

## 4. Salinomycin's effect on CSC stemness

Studies on other types of cancer have demonstrated salinomycin can overcome many characteristics that make CSCs difficult to treat. ABC transporters export many drugs out of the cell, thus decreasing their intracellular concentration and potency (42,43). This allows CSCs to survive many commonly administered chemotherapy agents. However, Fuchs *et al* discovered salinomycin is able to overcome these transporters and remain effective against leukemia stem cells (25). Another important CSC characteristic is invasiveness. CSCs are believed to play an important role in metastasis, the deadliest cancer progression. Multiple researchers have shown salinomycin decreases CSC invasiveness and migration (44-47). This physiologic change is associated with the FAK-ERK1/2 signaling pathway in liver CSCs (44) and the abolition of STAT3 and STAT1 interactions in colorectal CSCs (45). Salinomycin has also been shown to differentiate CSCs, transforming them from their chemoresistant and tumorogenic state to a state that can be eliminated by common chemotherapeutics (46,48). While it is not known exactly how salinomycin modulates these CSC characteristics, numerous studies have shown the drug interferes with the Wnt, Notch, and Hedgehog signally pathways, all of which are important for CSC maintenance (20,49-52).

## 5. Mechanism of action on GBM

The three primary regulated cell death pathways are apoptosis, autophagic cell death, and necrosis (53,54). Apoptosis can be triggered either extrinsically or intrinsically and results in the activation of caspase-enzymes that breakdown the cell in a controlled manner that does not negatively impact the surrounding tissue (54-56). Autophagy on the other hand, is a pathway that can have pro-survival or pro-death effects (54,57,58). Autophagy can degrade organelles to provide energy for the cell, but over activation can lead to cell death. Necrosis is thought to be a more uncontrolled cell death which causes the release of cell contents into the extracellular environment leading to inflammation. However, recent studies have identified specific necrotic pathways leading to the idea of controlled necrosis (54,59). The effect of salinomycin on indicators for these three pathways in GBM is discussed here and summarized in Table I.

*Apoptosis*. Evidence of salinomycin's ability to induce apoptosis has been shown for leukemia, prostate cancer, breast cancer, and ovarian cancer (21,22,60,61). However, studies on GBM have not found such a consistent trend. Common methods used to assess apoptosis include Annexin V flow cytometry, pro-caspase-3 cleavage via western blotting, and caspase-3/7 activity. Of the four studies that analyzed Annexin V flow cytometry, Chen *et al* showed a 25% positive population with salinomycin treatment, while the other three studies showed no or minimal Annexin V staining (27,29,30,62). Western blot analysis of pro-caspase-3 cleavage was detected in Chen *et al* and Qin *et al* but not in Calzolari *et al*, Booth *et al*, or Xipell *et al* (27,29,30,62,63). Surprisingly, the amount of pro-caspase-3 cleavage detected in Chen *et al* was greater for the lower salinomycin concentration, calling into questing the validity of the data. Only Qin *et al* and Xipell *et al* analyzed caspase-3/7 activity. While Qin *et al* found an increase in activity, Xipell *et al* found no difference (27,30). Though, Qin *et al* found the cleavage of pro-caspase-3 and an increase in caspase-3/7 activity, they found apoptosis was not the primary contributor to cell death in their GBM cells. Only a

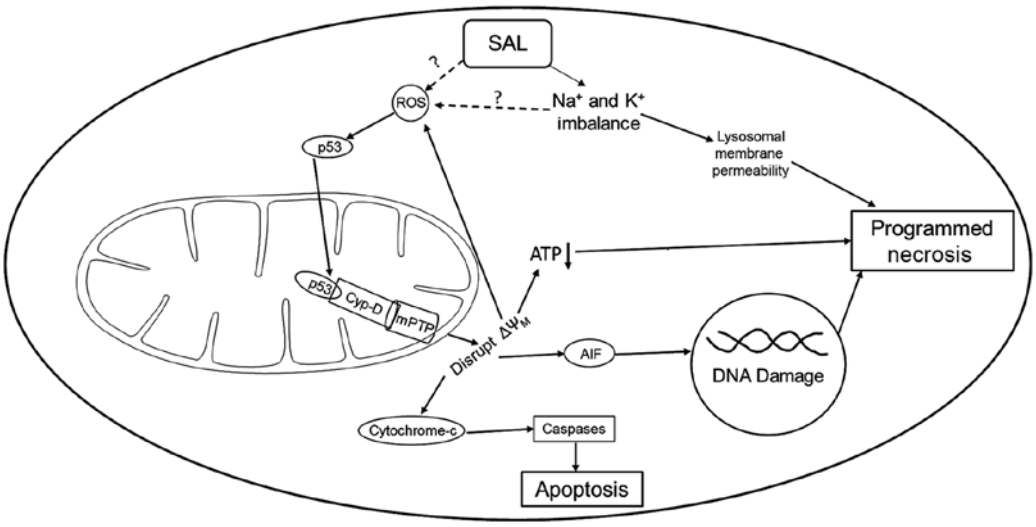


Figure 2. Summary of salinomycin's mechanism of action on GBM. Either a direct or indirect method causes ROS upregulation leading to p53 migration into the mitochondria. In the mitochondria, p53 forms a complex with Cyp-D to open the mitochondrial permeability transition pore, disrupting the mitochondrial membrane potential and leading to the release of cytochrome *c* and AIF. Cytochrome *c* activates the caspases causing apoptosis, while AIF damages DNA leading to programmed necrosis (27). The mitochondrial membrane disruption also decreases ATP production, adding another signal toward necrosis. In addition to upregulating ROS, salinomycin causes a $Na^+$ and $K^+$ imbalance which is hypothesized to interfere with lysosomal membrane permeability and also lead to programmed necrosis (30).

small part of the cell death was ameliorated using the apoptosis inhibitor zVADfmk (27). Together these data suggest apoptosis may play a role but is not the main mechanism of cell death of GBM. This is a promising conclusion for it suggests GBM are killed using a different mechanism than normal neuron and Schwann cells.

*Autophagic cell death*. The role of autophagy in salinomycin-induced GBM death has not been investigated to as great a degree as apoptosis. The studies that have looked into it though indicate it plays some kind of role. Xipell *et al* found a larger number of acidic vesicles in salinomycin treated cells and confirmed this finding with transmission electron microscopy images showing an increase in autophagosomes, autolysosomes, and lysosomes (30). Biochemically, they found an increased conversion from LC3-I to LC3-II, a marker for autophagosome synthesis (64). They also, however, found increased p62 accumulation, suggesting insufficient autolysosome degradation (30). Consistent with these findings, Booth *et al* also found upregulation of LC3-II and p62 upon treatment with salinomycin (63). These results are similar to those obtained when GBM is treated with the autophagy inhibitor Bafilomycin A1 (30). The combination of salinomycin and Bafilomycin A1 amplified these effects (30). Lysosomal maturation was found to be decreased by salinomycin as evidence by a decrease in the amount of active cathepsin B, which requires a low lysosomal pH (30). This lack of lysosomal maturation may be explained by the destabilization of Donnon potentials by salinomycin as a result of its action as an ionophore for $Na^+$ and $K^+$ (30). Xipell *et al* found ROS also plays an interesting role in autophagy. The ROS inhibitor NAC (N-Acetyl-cystein) reduced p62 accumulation without affecting LC3-II upregulation in salinomycin treated cells (30). This suggests salinomycin induced ROS may be an important cause of the aberrant autophagic response.

*Necrosis*. While apoptosis and autophagy are affected to some degree by salinomycin, the most compelling studies point to necrosis as the predominant mechanism of salinomycin induced GBM death. Xipell *et al* found salinomycin results in three of the most common executioners of necrosis: low levels of intracellular ATP, lysosome membrane permeability, and osmotic swelling (30). Qin *et al* showed a high salinomycin concentration causes over 40% of cells to stain positive for PI but not Annexin V, indicating necrotic cell death (27). Furthermore, Qin *et al* found the general necrosis inhibitor Necrostatin-1 was able to prevent most of the salinomycin-induced necrosis and viability reduction (27). The combination of Necrostatin-1 and the apoptosis inhibitor zVADfmk eliminated virtually all salinomycin-induced death suggesting necrosis and apoptosis are both active in salinomycin-induced cell death with necrosis playing the larger role.

Though specific necrosis pathways are still being understood, Vaseva *et al* identified a necrosis pathway in 2012 involving p53 opening of the mitochondrial permeability transition pore (mPTP), known as the mitochondrial permeability transition-driven regulated cell death pathway (MPT-driven RCD) (53,65-67). In this pathway, an increase in reactive oxygen species (ROS) causes unphosphorylated p53 to migrate into the mitochondrial matrix where it binds with cyclophilin D (Cyp-D, PPID) forming a p53-CypD complex. This complex stimulates mPTP opening causing a loss in the mitochondrial membrane potential and the release of cytochrome *c* and apoptosis-inducing factor, leading to necrosis. Three studies investigating salinomycin and GBM have discovered that salinomycin increases ROS (27,30,63) and three studies (two overlapping) have also found that salinomycin causes a deterioration of the mitochondrial membrane potential (27,30,62). Unlike the inconsistency of the apoptosis indicators, there are no studies that suggest salinomycin does not increase ROS or interfere with the

mitochondrial membrane potential. Qin *et al* demonstrated in a step-wise manner that salinomycin-induced necrosis proceeds through the same necrotic pathway as identified by Vaseva *et al* (27,65). By knocking out and then overexpressing Cyp-D, they showed its necessity for salinomycin-induced necrosis. Knockdown of p53 also prevented cell death and the formation of the p53-CypD complex identified using western blot. ROS inhibition with N-acetyl-L-cysteine reduced p53 translocation into the mitochondria as well as mPTP opening, indicating the importance of ROS in this pathway (27). The details of this mechanism are shown in Fig. 2.

From an analysis of the current research on salinomycin's effect against GBM, MTP-driven RCD appears to be the primary mechanism of action with a small amount of death attributed to apoptosis. This is a different mechanism than the calcium-induced apoptosis that kills neurons and Schwann cells, providing support for potential therapeutic applications in the future (38). However, two important questions remain: 1) how does salinomycin's structure or action as an ionophore lead to ROS and 2) why is salinomycin selective to GBM over normal tissue and GSCs specifically?

## 6. Combination therapy

Combination therapy is a method that can potentially be used to reduce the concentration of salinomycin required. This can help prevent the neuron and Schwann cell toxicity caused by higher doses of the drug. Delwar *et al* used salinomycin in combination with the alkylating chemotherapy agents temozolomide (TMZ), carmustine (BCNU), and lomustine (CCNU) in a two-phase treatment approach (68). Cells were treated with one of the alkylating agents for three weeks and then with a low concentration of salinomycin or vehicle control for ten weeks. While the cells in the wells treated with vehicle control remained alive and were able to regrow, the number of surviving cells was drastically reduced in the wells treated with salinomycin. Only 21% of TMZ/salinomycin treated wells contained any live cells, suggesting combination therapy with TMZ and salinomycin as a promising treatment for prolonging patient survival (68).

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and valproate have also been found effective against GBM in combination with salinomycin (62,63). TRAIL is a protein that binds to TRAIL receptor I or II in order to induce apoptosis, usually in tumor cells (69,70). Calzolari *et al* showed that combination of salinomycin and TRAIL resulted in 5-10% GBM viability while salinomycin alone resulted in 70% viability (62). The combination therapy leads to apoptotic death indicated by Annexin V/PI flow cytometry and pro-caspase-3 cleavage. Interestingly, the apoptosis inhibitor zVAD was able to prevent the significant viability reduction in only one of the three cell lines examined. Salinomycin was found to upregulate TRAIL-R2 leading to the increased potency of the combination (62). Valproate is a histone deacetylase inhibitor commonly used to treat epilepsy and bipolar disorder (71). When combined with valproate, only a low salinomycin concentration was required to induce significant cell death (63). Valproate reduced the autophagic effects caused by salinomycin, preventing LC3-II and p62 accumulation. The combination resulted in an upregulation of ROS, the suppression of which reduced cell death (63). Overexpression of the caspase-8 inhibitor c-FLIP-s and knockdown of the death receptor CD95 both significantly reduced the combination's toxicity (63). The ability of TRAIL and valproate to increase salinomycin's toxicity will allow a lower dose of salinomycin to be used, leading to potential clinical benefits.

## 7. Conclusion

Glioblastoma multiforme is the most common and deadliest form of primary brain tumor. It is heterogeneous in nature, containing a subpopulation of cells known as GSCs which are resistant to chemotherapy and radiation (1,2). In order to effectively treat GBM, these cells must be eliminated. Salinomycin has shown efficacy in treating other types of cancer stem cells and the studies that have been conducted on GBM have shown signs of GSC depletion including a greater sensitivity of GSC-enriched cultures (29,30), decreased neurosphere formation (27,29,30), and decreased GSC markers detection via qRT-PCR (30). However, this evidence is insufficient to prove that salinomycin targets GSCs. To confirm this hypothesis, future research should look more extensively at the effect of salinomycin on protein and gene expression as well as examine the tumor-seeding ability of salinomycin-treated cells.

It is not only important that salinomycin can kill GSCs, but also that it does not harm normal neural cells. Neural toxicity has been shown in cats, dogs and even humans in case studies (34,36,37). In 2011, Boehmerle *et al* demonstrated that this mechanism of death is through the calcium-induced apoptotic pathway and can be prevented by $Na^+/Ca^{2+}$ exchanger (NCX) inhibitors (38). In order for salinomycin to still be a useful GBM treatment, GBM and GSCs specifically must either 1) be more sensitive to calcium-induced apoptosis or 2) be killed through a different, GSC-specific, mechanism. This review found that salinomycin likely acts through a GSC-specific mechanism, with most death caused by MPT-driven RCD, with apoptosis playing a lesser role. To confirm this hypothesis, GBM NCXs should be either inhibited or knocked out and salinomycin toxicity determined. If salinomycin is able to still kill GSCs in the absence of working NCXs, then combination treatment with salinomycin and an NCX inhibitor is a promising future treatment regimen for GBM. Salinomycin would kill GSCs specifically via MPT-driven RCD while the NCX inhibitor would prevent salinomycin from harming normal neurons through the calcium-induced apoptotic pathway.

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