Chapter

A Role for Cardiac Glycosides in GBM Therapy

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Abstract

There is a pressing need for new effective therapeutic strategies to treat glioblastoma (GBM). Cardiac glycoside compounds consisting of both cardenolides and bufadienolides have been shown to possess potent activity against GBM cell lines and in vivo GBM tumors. In addition, recent research has shown that certain cardiac glycoside compounds contribute to an additive and even synergistic manner with the standard of care GBM treatments such as radiotherapy and chemotherapy. Finally, the finding that cardiac glycosides may offer a unique role in the control of GBM stem cells offers hope for better therapeutic outcomes in treating this deadly form of brain cancer.

Keywords: cardenolides, bufadienolides, digoxin, oleandrin, *Nerium oleander*, PBI-05204, glioblastoma, radiotherapy, stem cells, Na,K-ATPase

1. Introduction

While basic and clinical research has led to better diagnostic techniques and therapeutics for the treatment of glioblastoma, unfortunately, these have translated into only a modest improvement in median survival for this disease due to a high rate of recurrence [1]. As median survival for most GBM patients from time of diagnosis is less than 15 months, the need for new therapeutic approaches is clear [1–3]. Recent studies have shown that both cardenolide and bufadienolides compounds may offer a new therapeutic strategy for the treatment of GBM either as standalone compounds or in combination with other therapeutic modalities such as radiotherapy or standard of care drugs such as temozolomide. One cardenolide compound, oleandrin, derived from *N. oleander*, has shown particular promise against human GBM tumors both in vitro and in vivo. Oleandrin is a good addition to radiotherapy and certain chemotherapeutic agents such as temozolomide. In addition, this molecule and extracts containing it, such as PBI-05204, have now been shown to provide valuable activity against GBM stem cells which, in large part, account for the treatment of resistance and disease recurrence.

2. Cardiac glycosides and GBM

Chemically, cardiac glycosides can be divided into two groups: cardenolides and bufadienolides. Bufadienolides include bufalin, gammabufotalin, marinobufagenin,

and proscillaridin while common cardenolides include digoxin, digitoxin, ouabain, lanatoside C, and oleandrin [4, 5]. The action of members of both classes of compounds are known to have a role as therapeutic compounds for the treatment of congestive heart failure and, over the past decade, many of these compounds have also been reported to have the potential to treat a variety of human malignant diseases [6–9]. While high doses of cardenolides are frequently associated with cardiotoxicity, lower doses are still used for the treatment of congestive heart failure [10, 11]. In addition, low concentrations of selected cardenolides, such as oleandrin, are known to activate a precise signaling pathway or signalosome involving α-subunits of Na,K-ATPase and acting via Src-EGFR-Ras–Raf-extracellular signal-regulated kinase (ERK), Akt/Protein kinase (PK)B, and phosphoinositide 3-kinase (PI3K) to inhibit cell proliferation and survival [12, 13]. Proteomic profiling reveals upregulated PI3K-Akt–mTOR signaling across brain metastasis histology [14]. Given these are pivotal oncogenic factors for various malignancies, this represents an important new approach to the treatment of cancer. While the majority of published studies cite in vitro activity against key cancer cell lines, some, such as PBI-05204 (an extract of *N. oleander* containing oleandrin as a key active ingredient), have advanced to Phase I and II clinical trials for the treatment of patients with cancer [15, 16]. Importantly, constituents of both classes of cardiac glycoside compounds have shown promise as potential novel therapeutic agents for the treatment of GBM [17–28]. This is further supported by the result of our systematic repurposed drug screening to discover an effective therapeutic approach for the treatment of medulloblastoma. By applying a systemic biological approach including driver signaling network identification and drug functional network-based drug repositioning, we screened more than 1300 drug candidates. Among the 100 drugs predicted to be the most effective for the treatment of group 3 and 4 medulloblastoma, five cardiac glycosides including both cardenolides and bufadienolides were identified as having great potential to inhibit the growth of Group 3 and 4 medulloblastoma, which augments the therapeutic potential of cardiac glycosides in GBM [29].

2.1 Bufadienolides and GBM

Chansu is a traditional Chinese medicine and has been used for many years as a treatment for cancer. Bufalin, an active component of Chansu, is a naturally occurring compound classified as a bufadienolides and has been recognized as a specific inhibitor of Na, K-ATPase [21]. This compound has been shown to have antitumor activity against various cancers, such as liver, lung, intestinal, gastric, gynecological, and pancreatic [30]. Lan et al. point out that the sodium pump α-1 subunit of Na, K-ATPase regulates bufalin sensitivity of human glioblastoma cells through the p53 signaling pathway [20]. A novel observation by these researchers indicated that bufalin inhibits glioblastoma growth by promoting proteasomal degradation of the Na, K-ATPase α-1 subunit [31]. Additional mechanistic studies confirmed the important roles of Src and p53 signaling in mediating apoptosis. Importantly, bufalin inhibited the growth of glioma xenografts. The authors concluded that therapies targeting specific Na^+, K^+-ATPase α subunits such as α-1 and p53 signaling-mitochondrial apoptotic pathways may have the potential to treat gliomas [31].

A related bufadienolides compound, gamabufotalin, is another component of the traditional Chinese medicine Chansu and its pharmaceutical formula known as Huachansu. Yuan et al. have shown that gamabufotalin exhibited selective cytocidal effects against intractable cancer cells including glioblastoma, but minimal effects on

normal peripheral blood mononuclear cells prepared from healthy volunteers [22]. Additionally, they also reported that gamabufotalin efficiently downregulated the percentage of $CD4^+CD25^+Foxp3^+$ regulatory T (Treg) cells, which have been considered to play a critical role in limiting antitumor immune response in the body and promoting immunological "ignorance' of cancer cells [32]. Recent research by Yuan et al. have shown that treatment of the human glioblastoma cell line U-87 with gamabufotalin produced downregulation of the expression of uPA, CA9, and upregulated the expression of TIMP3, all of which are associated with invasion/metastasis. They conclude that this molecule exhibits significant cytotoxicity against cancerous glial cells with high potency and selectivity through multiple cytotoxic signaling pathways [22].

A related bufadienolide, marinobufagenin (MBG), has also been reported to be able to inhibit glioma growth through its ability to bind to the sodium pump α-1 unit and interaction with the ERK signaling mediated mitochondrial apoptotic (MAPK/ERK) pathway [33]. MBG treatment of U87MG and U251 cells markedly inhibited α-1 subunit expression. The effect of MBG to inhibit U251 xenograft subcutaneous growth was also assessed. Mice were treated with MBG for 9 days after which tumor volume and weights were assessed. These determinations showed significant inhibition of tumor growth resulting from MBG treatment. In addition, immunohistochemical analysis of tumor tissue demonstrated a significant decrease in the activated form of p65. Taken together, the authors stated that their results indicate that MBG effectively inhibits glioma growth through ERK-mediated mitochondrial apoptotic pathways [33]. Furthermore, MBG was observed to inhibit activation of NF-κβ and expression of other proinflammatory mediators including iNOS, COX-2, TNF-α, and IL-6 suggesting anti-inflammatory activity.

Proscillaridin is a cardiac glycoside that is derived from plants of the genus Scilla and *Drimia maritima*. Denicolai et al. [28] used two human primary GBM stem cell lines (GSCs), GBM6 and GBM9 in addition to the regular GBM cells to investigate the relative potential of proscillaridin to inhibit cell growth both in vitro and in vivo. The chosen cell lines are interesting in that GBM6 cells are highly malignant whereas GBM9 cells exhibit a much lower migratory capability yet have a higher proliferation rate [28]. Proscillaridin A exerted both anti-proliferative and anti-migratory activities in these cell lines at a concentration of 0.05 μM. The authors stated that proscillaridin was more active than temozolomide which in their study did not affect the migration or proliferation rate of either GBM6 or GBM9 cells. Exploring likely mechanisms of action for proscillaridin, the authors reported that this compound induced concentration-dependent cytotoxicity through both an increase in GBM cell death and a G2/M cell phase arrest thereby impairing a GBM stem self-renewal capacity.

2.2 Cardenolides and GBM

2.2.1 Digoxin

Of all the known cardenolide compounds the most widely studied is digoxin which in the recent past was widely used for the treatment of atrial fibrillation. Its potential activity as a repurposed drug for the control of GBM, however, is less well-known. Papale et al. [34] have examined the potential role of digoxin in the control of adverse effects of GSCs. They hypothesized that GSCs express receptors that can bind alarmins released during necrosis, an event favoring GSCs migration. Alarmins are endogenous molecules that are constitutively available and released upon tissue damage and activate the immune system. Uncontrolled and excessive release of alarmins is

believed to contribute to dysregulated processes seen in many inflammatory conditions such as tumorigenesis and tumor metastasis [35]. To investigate this hypothesis, GSC cell lines were kept under hypoxic conditions to determine the expression of hypoxic markers as well as receptors for advanced glycation end products. The authors reported that necrotic extracts increased migration, invasion, and cellular adhesion. Importantly, HIF-1α inhibition by digoxin prevented the response of GSCs to hypoxia. They concluded that inhibition of hypoxic pathways may represent a target for preventing brain invasion by glioblastoma stem cells [34]. Hypoxia and necrosis, with subsequent microenvironment inflammation, can be considered as two main features of growing GBM tumors and thus are believed to play a major role in determining the metastatic potential of GSCs in a tumor. The potential role of a cardenolide such as digoxin as an inhibitor of HIF-1α is intriguing as it may represent a novel means of inhibiting this master regulator in the complicated process of cellular adaptation to tumor microenvironments.

A related study of the role of hypoxia with regard to its potential to increase the expression of stem cell markers and promotion of clonogenicity of glioblastoma neurospheres was undertaken by Bar et al. [36]. They examined the effect of hypoxia on stem-like cells in glioblastoma using GBM-derived neurosphere cultures. When these were grown in 1% oxygen, HIF-1α protein levels increased dramatically as did mRNA encoding other hypoxic response genes, such as hypoxia-inducible gene-2, lysyl oxidase, and vascular endothelial growth factor. The rise in the stem-like fraction in GBM following hypoxia was paralleled by a two-fold increase in clonogenicity. The authors examined the potential of digoxin to prevent hypoxic-related events. They observed that this cardenolide suppressed HIF-1α protein expression, HIF-1α downstream targets, and slowed tumor growth in vivo. In addition, their data demonstrated that pretreatment with digoxin reduced GBM flank xenograft growth of hypoxic GBM cells. Daily intraperitoneal injections of digoxin were reported to have significantly inhibited the growth of established xenografts and suppressed the expression of vascular endothelial growth factors [36].

As stated earlier, we have shown that systemic in vivo treatment of patient-derived orthotopic xenograft (PDOX or orthotopic PDX) models of groups 3 (ICb-2555 MB) medulloblastoma that harbors c-Myc amplification and group 4 (ICb-1078 MB, that harbors an n-MYC amplification) medulloblastoma with digoxin, a member of cardiac glycoside approved for the treatment of heart failure, significantly prolonged animal survival times at plasma concentrations known to be tolerated in human. The antitumor effect of digoxin in medulloblastoma appears to be mediated by the down regulation of the Erk and Akt signaling pathway [29].

2.2.2 Digitoxin

Digitoxin is a cardiac glycoside similar in structure and effects to digoxin, though the effects are longer-lasting. This drug has been used to treat pain and inflammation associated with various diseases such as arthritis, AIDS, and atherosclerosis [23, 37, 38]. Studies have also shown that digitoxin induces growth inhibition and/or apoptosis of a variety of human cancer cells in vitro and in vivo [29]. Lee et al. examined the potential sensitizing effects of digitoxin and tumor necrosis factor-related ligand (TRAIL)-mediated apoptosis in human glioma cells [23]. TRAIL, a member of the tumor necrosis factor family, can bind to death receptors (DR4 or DR5) leading to oligomerization of the receptor's intracellular death domains and then to the recruitment of the adaptor molecule. Fas-associated death domain protein, and activation of caspases 3

and 8 [23]. However, an obstacle to effective therapy is the development of resistance to TRAIL by brain tumors. The research conducted by Lee et al. presented evidence that a combination of non-apoptosis inducing concentrations of digitoxin and TRAIL led to apoptosis of human glioma cells. Furthermore, they showed that the upregulation of DR5 expression and downregulation of the expression of survivin synergistically enhanced TRAIL-induced apoptosis by digitoxin in human glioma cells [23].

In a more recent article, researchers examined the sensitizing effects of digitoxin to TRAIL-induced apoptosis in GSCs cultured in vitro. They reported that the combination of TRAIL and digitoxin led to apoptosis of GSCs and an upregulation of DR5 expression in addition to down-regulation of surviving expression [24].

2.2.3 Ouabain

Ouabain, also known as g-strophanthin, is a plant-derived cardenolide that has in the past been used as an arrow poison in Africa. However, it has also been more traditionally used to treat hypotension, congestive heart failure, and some arrhythmias [39]. Interestingly, ouabain is also an endogenous molecule found in animals and humans during normal conditions and increases in concentration in response to high salt intake [40]. It has been reported that ouabain can activate multiple protein kinases such as MAPK, PKC, and phosphoinositide 3-kinase (PI3k)/Akt by binding to Na,K-ATPase [25] and that this is part of the anticancer mechanisms of this molecule. Yan et al. noted that some of these pathways are involved in p66hc phosphorylation [25] suggesting to them that ouabain-induced reactive oxygen species (ROS) was involved.

They examined the intracellular changes induced by ouabain in human glioblastoma cells and noted that prior reports of ouabain-induced mitochondrial membrane loss and elevated ROS production were associated with human cancer cell apoptosis. In a set of interesting experiments, these investigators showed that ROS was increased in glioblastoma cells exposed to ouabain, however, this was not due to calcium overload. Rather, it appears to be the result of p66Shc phosphorylation as part of the Src/Ras/ERK signal pathway [25].

Yang et al. also explored mechanisms of ouabain-mediated cell death of glioblastoma cells. Compared to untreated U-87MG cells, ouabain suppressed survival and attenuated cell motility in a concentration-dependent manner. In addition, they observed downregulation of p-Akt, mTOR, p-mTOR, and HIF-1α at low concentrations of ouabain. The authors suggest that these results indicate that ouabain exerted suppressive effects on tumor cell growth and motility, leading to cell death via regulating the intracellular Akt/mTOR signaling pathway and inhibiting the expression of HIF-1α in glioma cells [41].

2.2.4 Lanatoside C

Lanatoside C is an antiarrhythmic agent, a naturally occurring compound extracted from *Digitalis lanata*. Badr et al. reported that this cardenolide is a sensitizer of GBM cells to TRAIL-induced cell death partly by upregulation of the death receptor 5. This was evident in GBM cells in culture as well as in a GBM xenograft model in vivo [26]. Cells treated with lanatoside C showed necrotic cell morphology with the absence of caspase activation, low mitochondrial potential, and early intracellular ATP depletion. This suggests mitigation of apoptosis resistance by inducing an alternate cell death pathway. The combined treatment was highly effective as a

low dose of lanatoside C sensitized GBM cells to TRAIL in culture killing over 90% of U87GBM cells, while it had no significant effect on primary fibroblasts [26]. The authors pointed out that to use the suggested combination of TRAIL with lanatoside C in vivo, there would have to be a means of delivering TRAIL intracerebrally.

In follow-up articles, the lab of Bakhos Tannous, PhD (Massachusetts General Hospital) used an adeno-associated virus (AAV) vector specifically designed for intracranial delivery of secreted, soluble tumor necrosis factor-related apoptosis-inducing ligand (sTRAIL) to GBM tumors in mice. They combined the AAV delivery vehicle with the TRAIL-sensitizing cardenolide, lanatoside C. This unique combination was applied to two different GBM models using human U87 glioma cells, primary patient-derived GBM neural spheres in culture and orthotopic GBM xenograft models in mice. The authors correctly point out that a major pitfall in testing new GBM therapeutics is the use of animal models that do not accurately recapitulate a phenocopy of the human tumor [42, 43]. Typical cell lines such as U87 form local tumors that do not invade the brain per se. Therefore, the investigators tested the AAV-sTRAIL and lanatoside C therapy using primary cells dissociated from GBM patient specimens and grown as stem-like neural spheres which invade the mouse brain upon intracranial injection which replicates that occurring in the original tumor [43]. Despite the ingenuity of this therapeutic approach both the single and multi-injection approach of sTRAIL combined with lanatoside C showed only a modest survival benefit with animals eventually succumbing to the disease.

2.2.5 UNBS1450

UNBS1450 is a hemi-synthetic cardenolide belonging to the cardiac steroid glycoside family. The molecule has been shown to induce apoptotic cell death in malignant cells. It inhibits NF-kβ transactivation and triggers apoptosis by cleavage of pro-caspases 8, 9, and 3/7, by decreasing expression of anti-apoptotic Mcl-1, and by recruitment of pro-apoptotic Bak and Bax proteins [44]. UNBS1450 has been tested in 58 distinct human cancer cell lines and displays antitumor effects similar to Taxol [45]. It has also been reported to be active on Taxol-resistance cell lines. Of particular interest was the observation that this semi-synthetic cardenolide demonstrated antiproliferative effects against three glioblastoma cell lines with a level of activity similar to vincristine but much greater than those displayed by temozolomide, tamoxifen, hydroxy-tamoxifen, lomustine, procarbazine, and carmustine [46, 47]. The ability of UNBS1450 to be especially effective against glioblastoma cell lines can be explained, in part, by the fact that U373-MG GBM cells express a higher level of Na, K-ATP α-1 subunits than normal cells which is a particular target for this molecule. Similar to other cardenolides, UNBS1450 also decreases the intracellular ATP concentration more markedly in glioblastoma cells than in normal cells [47]. The advanced feature of this compound is that it can inhibit three isoforms (α3β1, α2β1, and α1β1) with relatively higher efficiency (~6 to >200 times) than ouabain and digoxin [47]. While UNBS1450 was tested in clinical trials using a dose-intensification study to find the MTD, toxicity, and pharmacokinetic parameters of the molecule in patients with lymphoma, the clinical trials were unfortunately closed by the sponsor before reaching the MTD in patients [5].

2.2.6 Oleandrin

Oleandrin is a highly lipid-soluble cardenolide isolated from the plant *N. oleander* and has been used as a traditional herbal medicine due to its excellent

pharmacological properties [38]. Like other cardenolides, oleandrin has been used for the treatment of congestive heart failure; however, more recently oleandrin has attracted attention due to its extensive anti-cancer and novel anti-viral effects. In vitro and in vivo investigations have shown that oleandrin possesses anticancer properties against several cancers including melanoma, leukemia, sarcoma, prostate, lung, pancreatic, and brain cancers [5–7, 12, 13, 17, 38]. Mechanisms underlying the anticancer activity of oleandrin include cell cycle arrest [48], altered membrane fluidity [49], modulation of cell signaling pathways (NF-kβ, JNK) [50], elevated Ca^{2+} and Na^{+} levels, decreased K^{+} levels inside the cell [51, 52], oxidative and mitochondrial stress [53], altered IL-8 levels [54], reduced expression of Rad51 [55], and decreased activation of fibroblast growth factor-2 [56]. Defined extracts of *N. oleander* containing this molecule (i.e., Anvirzel™ and PBI-05204) have undergone clinical trials in cancer patients where both the relative safety and pharmacokinetics of oleandrin were determined [15, 16, 57].

Of particular interest is the ability of oleandrin to act as a chemosensitizer for both chemotherapeutic and radiotherapeutic strategies. The development of resistance to drugs and radiotherapy is a major hurdle toward the effective treatment of cancer [58]. Oleandrin has been shown to reduce radiotherapy resistance in triple-negative breast cancer cells [59] and was also shown to sensitize human prostate cancer cells to radiotherapy [60]. It has also been indicated to exhibit significant antitumor effects in radiotherapy resistant MDA-MB231 cells which was reported to be due to inhibition of phosphor-STAT3, reduced levels of OCT3/4, β-catenin, and decreased MMP-9 activity [59]. These results have been suggested as important with respect to breast cancer invasion. Additionally, various studies have shown that oleandrin decreases tumor size and tumor development and inhibits cellular proliferation in human or murine glioma cells by increasing brain-derived neurotrophic factor (BDNF) levels, decreasing tumor infiltration, and reducing angiogenesis. It was also concluded that oleandrin can be used in adjuvant therapy with currently available chemotherapeutics such as temozolomide.

Oleandrin and extracts that contain this molecule may have unique abilities for the effective treatment of GBM. Digoxin is actively excluded from the brain via P-glycoprotein, yet oleandrin efficiently crosses the blood–brain barrier and inhibits P-glycoprotein expression [17, 61]. Lin et al. investigated 12 human tumor cell lines to explore pathways of tumor cell sensitivity to cardenolide compounds [62]. In vitro models of human glioma included HF U251 cells as well as native and modified melanoma BRO cells. A study by Lefranc and Kiss suggested that high expression of Na,K-ATPase α1 isoform in the presence of low α3 expression was associated with relative sensitivity to cardiac glycosides such as oleandrin, ouabain, and bufalin [63]. Other investigators, however, have found that the higher the Na, K-ATPase α3/α1 ratio, the greater the sensitivity to oleandrin [64].

Garofolo et al. examined the effects of oleandrin on glioma models in vivo [13]. They inoculated human glioma cells into mice and investigated the antitumor efficacy of oleandrin. Administration of this cardenolide reduced glioma growth and lowered cell proliferation. Furthermore, in a recent review of the potential of oleandrin to treat glioblastoma [17], the authors point out that oleandrin increases the cerebral levels of brain-derived neurotrophic factor (BDNF), decreases both microglia/macrophage infiltration and CD68 immunoreactivity in tumors, lowers astrogliosis in the tumoral penumbra, and attenuates glioma infiltration into healthy parenchymal tissue. In BDNF-knock out mice (bdnftm1Jae/J) and Trk-silenced glioma cells, the efficacy of oleandrin was diminished indicating a key role for BDNF in oleandrin's

antitumor efficacy. Garofalo et al. [65] had previously shown that BDNF inhibited the chemotaxis of glioma cells by blocking the small G-protein RhoA through the truncated TrkB.T1 receptor and that BDNF infusion reduced glioma volume in mice. Additionally, oleandrin was also shown to enhance survival in glioma-implanted mice increasing the efficacy of temozolomide [13].

Colapietro et al. recently reported the efficacy of PBI-05204 (a defined extract of *N. oleander* containing oleandrin as a principle active ingredient) in inhibiting the growth of human glioblastoma. Their studies were designed to investigate the antitumor efficacy of this botanical drug against glioblastoma using both in vitro and in vivo cancer models as well as exploring its efficacy against glioblastoma stem cells. They reported that three human GBM cell lines, U87MG, U251, and T98G were inhibited by PBI-05204 in a concentration-dependent manner that was characterized by induction of apoptosis as evidenced by increased ANNEXIN V staining and caspase activities [66]. An important clue to the mechanisms of anti-glioma growth was the finding that the expression of proteins associated with both Akt and mTOR pathways was suppressed by PBI-05204 in all three cell lines. PBI-05204 significantly suppressed U87 spheroid formation and the expression of important stem cell markers such as SOX2, CD44, and CXCR4. Oral administration of PBI-05204 to nude mice resulted in a dose-dependent inhibition of U87MG, U251, and T98G xenograft growth. Additionally, PBI-05204 treated mice carrying U87-Luc cells as an orthotopic model exhibited significantly delayed onset of tumor progression and significantly increased overall survival. Immunohistochemical staining of xenograft tumor sections revealed declines in Ki67 and CD31 positively stained cells but increased TUNEL staining. Given the fact that PBI-05204 has already been in phase I and II clinical trials for cancer patients, the authors concluded that further examination of the role of PBI-05204 in GBM patients should be considered [66].

2.3 Combination therapies with cardiac glycoside compounds

Cardiac glycosides represent a class of compounds that work well together with both drugs and radiotherapy in models of GBM. This effective combination of therapeutic strategies has been shown for both bufadienolides and cardenolide compounds. With respect to bufalin, for example, Zhang et al. investigated the response of U251 and U87MG glioblastoma cell lines. Bufalin reduces cell proliferation in both cell lines and induced a G2/M cell cycle arrest [67]. They also observed that bufalin disrupted the mitochondrial membrane potential leading to reduced oxygen consumption and ATP production. In addition, homologous recombination efficacy, a measure of DNA repair, was reduced by ~40%. This was associated with increased γH2AX expression, a marker for the presence of DNA double-strand breaks. Bufalin was additive with radiation in the treatment of GBM cells in vitro. Cell death increased significantly under combination treatment compared to radiation treatment alone [67].

In a recent article, Colapietro et al. explored the role of PBI-05204 in models of human glioblastoma when combined with radiotherapy [58]. This study demonstrated that PBI-05204 treatment led to an increase *in vitro* the sensitivity of GBM cells to radiation in which the main mechanisms were the transition from autophagy to apoptosis, enhanced DNA damage, and reduced DNA repair after radiotherapy administration. The combination of PBI-05204 with radiotherapy was associated with reduced tumor progression as evidenced in both subcutaneous as well as orthotopic implanted GBM tumors. The authors state that, collectively, their results reveal that

PBI-05204 enhances antitumor activity of radiotherapy in preclinical/murine models of human GBM and again call for further exploration of the use of this botanical drug in combination therapies in clinical trials.

Cardiac glycoside compounds have also been reported to be able to add to the antitumor efficacy of chemotherapeutic compounds used to treat GBM. Gamabufotalin has been reported to promote temozolomide sensitivity in glioblastoma cells [68]. Both in vitro and in vivo studies were undertaken to examine mechanisms to explain gamabufotalin's ability to increase sensitivity of GBM to temozolomide. Studies revealed a negative feedback loop between ATPA3 (α3 subunit of Na,K-ATPase) and AQP4 (aquaporin 4, a 'water channel' protein molecule), which were predicted by molecular modeling and docking studies to interact with gamabufotalin. The role of AQP4 in GBM growth and proliferation is an interesting finding in light of other studies showing that AQP4 knock out could play a role in several neurodegenerative diseases. Lan et al. reported that AQP4 suppression could significantly promote temozolomide sensitivity with the result that gamabufotalin might mediate inhibition of GBM via regulation of the ATP1A3-AQP4 signaling pathway [69].

In unpublished studies, we have also explored the in vitro and in vivo effects of oleandrin when combined with both radiotherapy and temozolomide in human glioblastoma cell models. Our studies extend a potential role of oleandrin and extracts that contain this molecule (e.g., PBI-05204) in combination with radiotherapy [67]. As radiotherapy and temozolomide are considered 'standard of care' treatment for GBM, any extension of their relative efficacy and success in clinical outcomes is indeed welcomed. Our preliminary studies have indicated again that the combined use of oleandrin with radiotherapy and temozolomide inhibited autophagy in favor of apoptotic pathways, reduced expression of NF-κβ, and reduced cell survival mechanisms while inducing DNA damage by suppression of Rad51. The combined treatments led to an increase in disease-free survival in mice with orthotopically implanted GBM tumors compared to either temozolomide or oleandrin treatment alone. Additional confirmatory studies are needed and are presently underway.

Furthermore, to enhance the translational potential of the therapeutic activity of oleandrin and extracts containing this compound (PBI-05204) in GBM, we evaluated the anti-proliferative effect of oleandrin in primary GBM cells isolated from human GBM-derived intra-cerebral (IC) orthotopic PDX models. We treated the three primary human GBM cell lines, IC-3704, IC-4687, and IC-3752 with different doses of oleandrin (1–100 or 1–1000 nM) and tested cell viability at 72 hrs after treatment. As shown in **Figure 1**, oleandrin exposure significantly inhibited the growth of all three GBM cells in a dose-dependent manner, with comparable low median inhibitory concentrations (IC_{50}) of 8.57, 9.73, and 6.02 nM for IC-3704 (**Figure 1A**), IC-4687 (**Figure 1B**) and IC-3752 (**Figure 1C**), respectively. To further test the antitumor efficacy of oleandrin-containing extract (PBI-05204) on human GBM tumors, we evaluated the overall survival of mice bearing the human GBM-derived IC orthotopic PDX tumor. The IC-1406 GBM was established through direct injection of surgical tumor specimens into mouse cerebrum areas [70]. The tumor was collected from a patient with a diagnosis of Turcort's syndrome carrying a c.137G > T (p.546 l) in the PMS2 gene and this mutation was present in orthotopic tumors. The IC-1406 GBM cell orthotopic model was developed by injecting these particular cells (1×10^5) into the right cerebrum. Treatment with PBI-05204 (25 mg/kg, qd × 5 days) was started at 2 weeks post-tumor cell injection. Analysis of median survival times of mice bearing IC-1406 GBM tumor was significantly

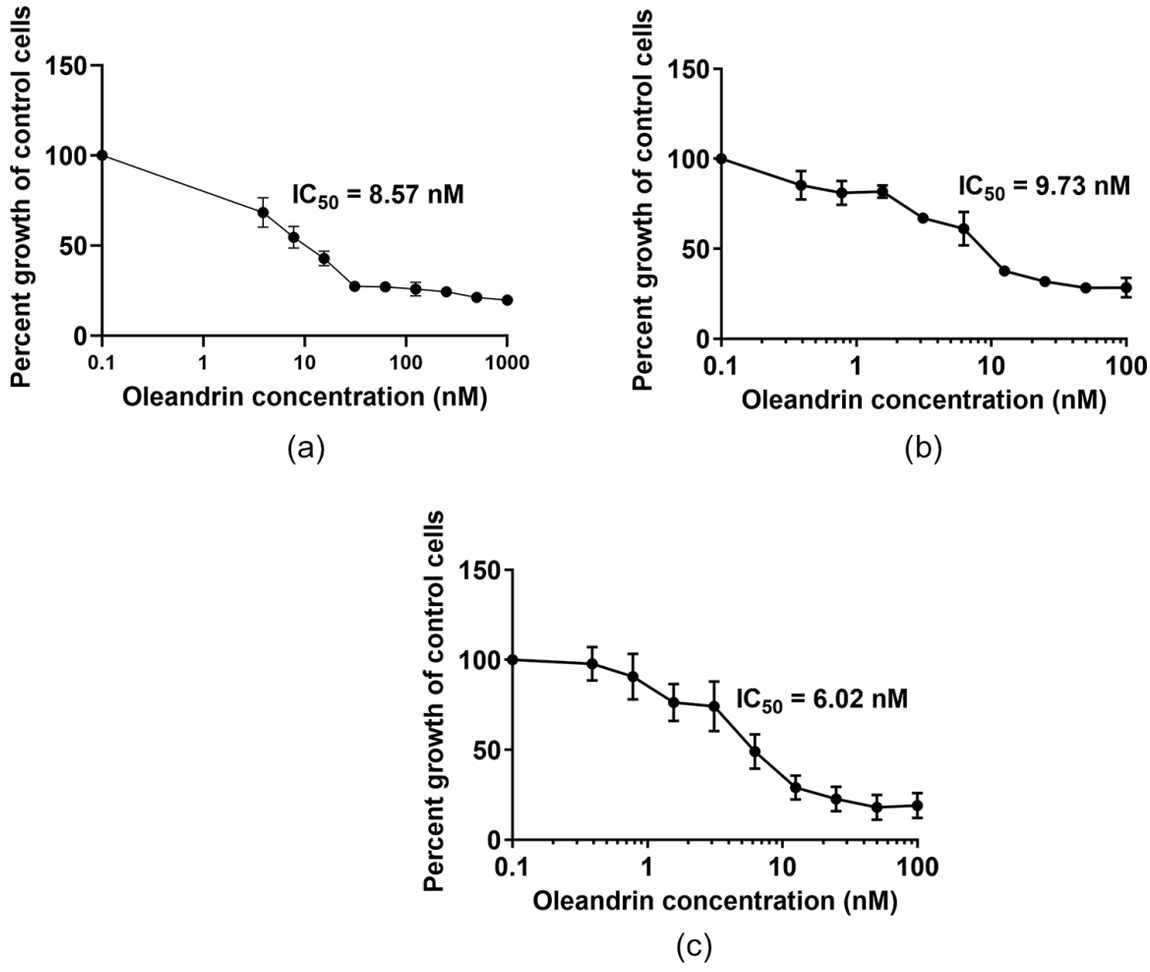

Figure 1.
Growth curves of human GBM cells derived from a human orthotopic PDX model. Primary cultured cells from IC-3704GBM (a), IC-4687GBM (b) and IC-3752GBM (c) cells (6 × 10^3) were plated and allowed to attach for overnight. They were then treated with oleandrin (1–100 nM) for 72 hrs. Cell proliferation was assessed by MTT assays. Data are presented as mean ± SD.

increased from 90 days in the control group to 122 days in the PBI-05204 treated group ($p < 0.006$) (**Figure 2A**), suggesting oleandrin and PBI-05204 exert strong antitumor efficacy in PDX derived GBM cells and their orthotopic model. While we had reported previously that PBI-05204 enhanced the antitumor efficacy of radiotherapy using established human GBM cell lines, such as U87MG, U251, and TG98 cell line mouse xenograft models, we then examined the possibility that PBI-05204 may have significant sensitizing effects on radiotherapy using a patient-derived orthotopic GBM PDX model IC-1128GBM [71]. As shown in **Figure 2B**, the combination of radiotherapy (XRT, 2 Gy × 5) and PBI-05204 resulted in a significant enhancement of overall survival compared to control or either single treatment modality alone. For example, the average overall survival of mice treated with PBI-05204 plus XRT was 158 days which was significantly longer than that in control mice (116 days, $p < 0.001$), PBI-05204 treated mice (118 days, $p < 0.001$), or XRT treated mice (144 days, $p = 0.022$), again suggesting PBI-05204 can enhance the antitumor efficacy of radiotherapy in GBM.

To understand the potential mechanisms involved in PBI-05204-elicited anti-tumor effects in the PDX derived GBM tumor, we examined the expression of cell cycle and apoptosis regulators as well as cell signaling proteins in tumor tissues collected from the IC-1406 PDX orthotopic model using Reverse Phase Proteomic

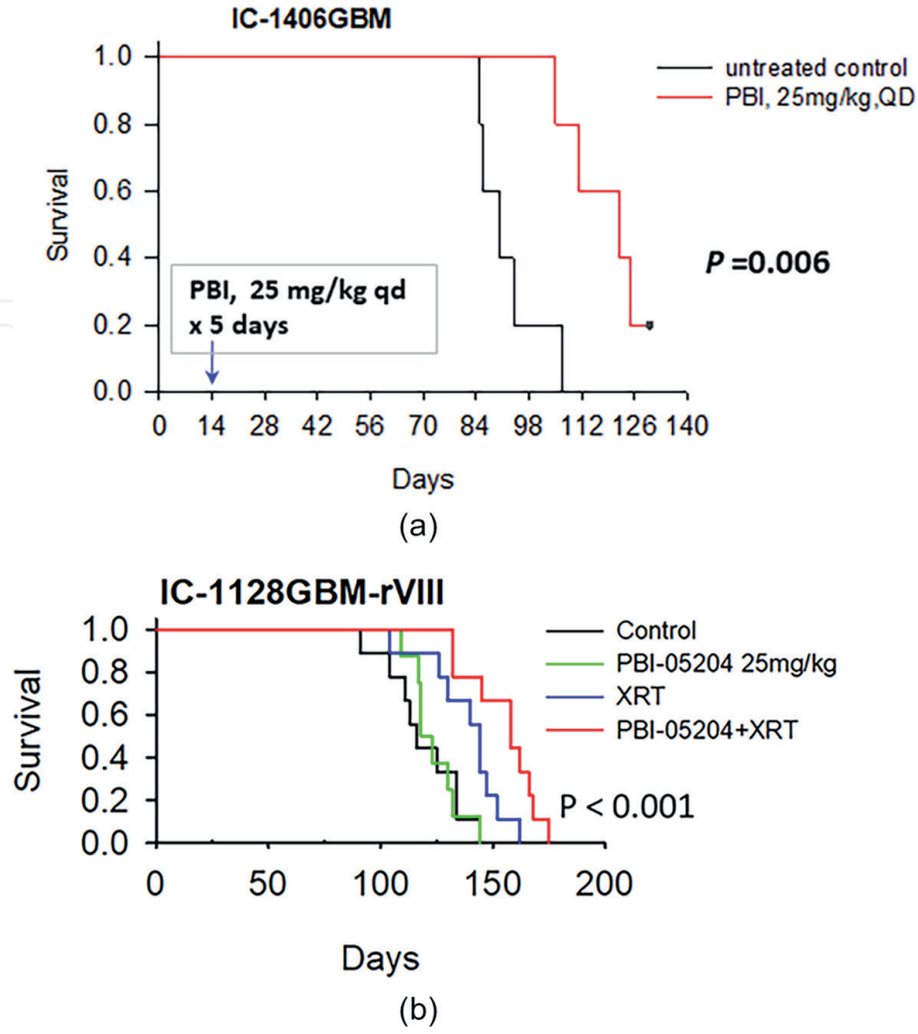

Figure 2.
Antitumor efficacy of PBI-05204 alone or in combination with radiotherapy in human GBM-derived intracerebral (IC) orthotopic PDX models. (a) Kaplan Meyer curves of mice bearing orthotopic PDX model of IC-1406 GBM treated with PBI-05204. (b) Kaplan Meyer curves of IC-1128 GBM derived PDX model at passage 8 (rVIII) after treatment with PBI-05204 (25 mg/kg), radiotherapy (XRT, 2 Gy/day × 5 days), or a combination of PBI-05204 and XRT.

Array (RPPA) analysis as performed by the Functional Proteomics Core Facility at The University of Texas MD Anderson Cancer Center. As shown in the Heatmap (**Figure 3A**), PBI-05204 treatment led to altered expression of several proteins associated with cell cycle, apoptosis, and oncogenic signaling pathway in the IC-1406 PDX model. Among these proteins, PBI-05204 BT significantly down-regulated SOX2 by 41%, an important stem cell marker presented in various cancer stem cells including GBM. Intriguingly, the abundance of tumor suppressor and negative regulator of PI3K/Akt pathway PTEN was significantly increased by PBI-05204 treatment by almost 2-fold. Consistent with this finding, the activity of a downstream target of PI3k/Akt pathways Ribosomal protein S6 was notably decreased by PBI-05204 evidenced by the phosphorylation of this protein was reduced in PBI-05204 treated tumor tissues compared to that of control mice (**Figure 3B**). These findings suggest that PBI-05204 can potentially inhibit the growth of GBM by upregulating PTEN and consequently downregulating the PI3K/Akt pathway and affecting cancer stem cells which were consistent with our previous study using the established human GBM cells.

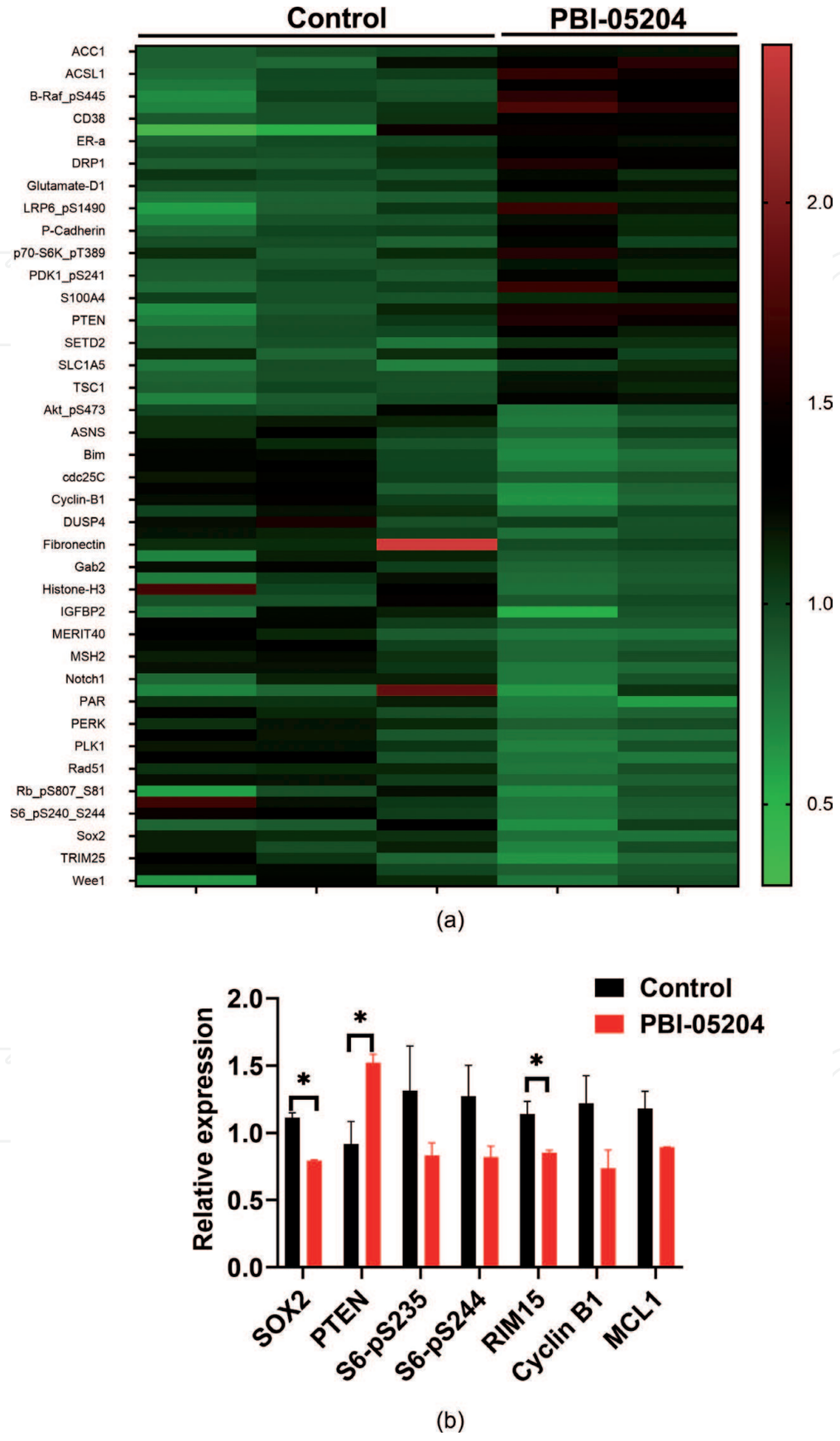

Figure 3.
*Proteomic analysis of tumor tissues derived from IC1406 PDX models by reverse phase proteomic Array (RPPA). (a) Heatmap of cell cycle regulating proteins and cell signaling proteins in PBI-05204 treated tumor tissues by RPPA. (b) Expression of cell cycle regulating proteins and cell signaling proteins showing about 20% changes following PBI-05204 treatment. Data are presented as mean ± SD. * $p < 0.05$ versus control.*

3. Cardiac glycosides and glioblastoma stem cells

Conventional treatment of GBM promotes a transient elimination of the tumor and, unfortunately, is almost always followed by tumor recurrence due to an increase in glioblastoma stem cell (GSC) populations [72]. It is believed that GSCs are the primary driving force behind GBM relapses. GSCs are typically resistant to further chemotherapeutic efforts and are typically resistant to additional radiotherapy [72]. To effectively eliminate GSCs, it is critical to target their essential functions and metabolism before effective strategies can be developed against them. While no single therapeutic modality has yet been shown to be completely effective against a heterogenous GSC population, recent studies have shown that cardiac glycosides may prove to have effective activity against GSCs and offer insights as to how they inhibit this specific cell population.

One important target that has been suggested as important for GSC proliferation is the hypoxia-inducible factor (HIF) family of transcriptional factors as they are master regulators of diverse cellular responses to hypoxic conditions. Among these, HIF1α plays a pivotal role in GBM survival, resistance, and invasion [73]. Nigim et al. [74] have reported a new orthotopic model of glioblastoma that recapitulates the hypoxic tumor environment of GBM tumors. This model is based on stem-like GBM cells that were isolated from a recurrent GBM. Their research demonstrates that digoxin is an effective inhibitor of HIF-1α expression and angiogenesis in vivo and provides survival benefits. Using the MGG123 model, the authors have shown that digoxin potently inhibits HIF-1α protein expression even after its induction with hypoxic conditions in vitro. Importantly, they also demonstrated digoxin-mediated HIF-1α silencing in orthotopic GBM xenografts. A related series of studies reported by Bar et al. demonstrated that digoxin also inhibits the growth of cultured GBM cells and flank GBM xenografts with concomitant reduction of HIF-1α and CD133 levels [36]. Thus, digoxin, and perhaps related cardiac glycosides, may effectively target HIF-1α, an important target against GSCs.

Proscillaridin A was shown to have cytotoxic and exhibit anti-migratory properties on GBM cell lines including stem-like cells, but not on healthy neural cells [28]. Berges et al. disclosed a novel pathway by which proscillaridin A and digoxin modulate microtubule network functioning in GBM and stem-like cells [27]. They found that at low concentrations proscillaridin A induced an alteration of microtubule dynamic instability. This was the result of GSK3β activation following the binding of proscillaridin binding to Na, K-ATPase, leading, in turn, to EB1 phosphorylation and subsequent inhibition of cell migration. They conclude that cardiac glycosides at low concentrations mimic the anti-migratory and cytotoxic effects of microtubule inhibiting drugs although they bind to Na, K-ATPase, and not directly to tubulin. As such, cardiac glycosides may represent an alternative treatment strategy and potent candidates for drug repositioning.

Many articles have cited the importance of cardiac glycosides targeting certain alpha subunits (e.g., α1 and/or α3) of Na,K-ATPase to combat the proliferation of glioblastoma cells, Li et al. [75] have indicated that targeting the β2 subunit of Na,K-ATPase represents a new approach to induce glioblastoma cell apoptosis through elevation of intracellular Ca^{2+}. The β-subunit is a glycoprotein involved in the structural maturation of Na,K-ATPase, and regulates enzyme stability, α-subunit activity, and cell adhesion processes. They point out that selectively targeting the β2 subunit that is not expressed in the heart might avoid cardiotoxicity. In contrast, the β2 subunit is more highly expressed in glioblastoma stem-like cells than in GBM cells. Its down-regulation selectively induces apoptosis in GSCs and is associated with significant inhibition of tumor growth in vivo.

Our own research has recently reported the effect of a defined extract of *Nerium oleander* containing oleandrin (PBI-05204) against human glioblastoma models and its ability to modulate GSC cell-renewal properties [67]. Three human GBM cell lines, U87MG, U251, and T98 associated with Akt and mTOR pathways were inhibited by PBI-05204 in a concentration-dependent manner that was characterized by induction of apoptosis as evidenced by increased ANNEXIN V staining and caspase activities. PBI-05204 significantly suppressed U87 spheroid formation and the expression of important stem cell markers such as SOX2, CD44, and CXCR4. Additionally, we also reported that when PBI-05204 was added to the irradiated GBM cells, it enhanced the antitumor efficacy of radiation in both GBM cells and their relevant animal models as well as significantly reducing the stemness of GBM cells. This was believed due to the down-regulation of CD44 and stro-1, an important mesenchymal stem cell marker in U87MG cells [58].

4. Conclusions

Cardenolide and bufadienolides compounds as well as semi-synthetic cardiac glycoside compounds such as UNBS1450 have now been shown to have potent activity against GBM cell lines as well as established in vivo tumor models. These compounds have been reported to have multiple mechanisms of action which are, in many cases, unique from those of conventional chemotherapeutic agents already approved as the standard of care drugs for GBM. It would thus appear that the combination of cardenolide or bufadienolides compounds with approved radiotherapy and chemotherapy (i.e., temozolomide) approaches to the treatment of GBM is an option worth exploring. Additionally, some cardenolides, such as oleandrin, are capable of crossing the blood–brain barrier and residing there in the brain (up to 24 hrs) longer than that in plasma providing an advantage of these compounds for the treatment of GBM. Finally, considering cognitive impairment is one of the major toxicities of radiotherapy and that some of these compounds, including neriifolin, oleandrin, and others, have been shown to exert neuroprotective effects [76], these compounds might not only be able to slow down the growth of GBM, but also provide a benefit assisting in the repair of radiation-induced damage to injured neurons. Some cardenolide compounds such as PBI-05204 containing oleandrin have already been through both Phase I and II clinical trials in cancer patients. Exciting new research has now clearly shown that this class of compounds also has potent activity in effectively reducing GBM stem cell populations known to be an important reason for the progression of disease after initial surgery and other therapeutic strategies have been performed.

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Conflict of interest

Robert A. Newman is the Chief Science Officer for Phoenix Biotechnology, Inc.; Peiying Yang is a consultant for Phoenix Biotechnology, Inc. All other authors claim no conflict of interest.

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