NEOPLASIA
www.neoplasia.com

Volume 16 Number 10 October 2014 pp. 874–882 874

Therapeutic Efficacy of Aldoxorubicin in an Intracranial Xenograft Mouse Model of Human Glioblastoma[1,2]

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Abstract

Glioblastoma multiforme (GBM) is the most aggressive primary brain tumor with a median survival of 12 to 15 months after diagnosis. Acquired chemoresistance, high systemic toxicity, and low penetration of the blood brain barrier by many anticancer drugs contribute to the failure of anti-GBM therapies. To circumvent some of these obstacles, we tested a novel prodrug approach to evaluate anti-GBM efficacy by utilizing serum albumin-binding doxorubicin (Doxo), aldoxorubicin (Aldoxo), which is less toxic, is released from albumin in an acidic environment and accumulates in tumor tissues. A human GBM cell line that expresses a luciferase reporter (U87-luc) was stereotactically injected into the left striatum of the brain of immunodeficient mice. Following initial tumor growth for 12 days, mice were injected once a week in the tail-vein with Aldoxo [24 mg/kg or 18 mg/kg of doxorubicin equivalents—3/4 maximum tolerated dose (MTD)], Doxo [6 mg/kg (3/4 MTD)], or vehicle. Aldoxo-treated mice demonstrated significantly slower growth of the tumor when compared to vehicle-treated or Doxo-treated mice. Five out of eight Aldoxo-treated mice remained alive more than 60 days with a median survival of 62 days, while the median survival of vehicle- and Doxo-treated mice was only 26 days. Importantly, Aldoxo-treated mice exhibited high levels of Doxo within the tumor tissue, accompanied by low tumor cell proliferation (Ki67) and abundant intratumoral programmed cell death (cleaved caspase-3). Effective accumulation of Aldoxo in brain tumor tissues but not normal brain, its anti-tumor efficacy, and low toxicity, provide a strong rationale for evaluating this novel drug conjugate as a treatment for patients afflicted with GBM.

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Abbreviations: GBM, Glioblastoma multiforme; Doxo, Doxorubicin; Aldoxo, Aldoxorubicin; HPLC, High-performance liquid chromatography; MTD, Maximum tolerated dose; TMZ, Temozolomide; BBB, Blood-brain barrier

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[1] Financial Support: Funding for this work was provided by CytRx Corporation to Om Prakash, and by National Institutes of Health (RO1-CA095518 and P20-GM103501) to Krzysztof Reiss.

[2] Disclosure of Potential Conflicts of Interest: Drs. Wieland and Levitt are employees of CytRx Corporation. The other authors have declared that no competing interests exist.

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Received 17 July 2014; Revised 22 August 2014; Accepted 25 August 2014

1476-5586/14

http://dx.doi.org/10.1016/j.neo.2014.08.015

Introduction

Glioblastoma multiforme (GBM) is the most aggressive primary neoplasm of the central nervous system (CNS) accounting for approximately 60% of all primary brain tumors with 12,500 new cases diagnosed in the US annually [1,2]. Standard of care for newly diagnosed GBM remains a multimodal regimen consisting of surgical resection with concomitant daily temozolomide (TMZ) and radiation therapy, followed by adjuvant TMZ [3]. Nevertheless, the increase in median survival is only 2.5 months compared with individuals treated with radiotherapy alone [3,4]. Additionally, acquired chemoresistance is a major problem with this therapy. Essentially all patients develop recurrent or progressive disease after the initial therapy showing no response to repeated challenges with TMZ [5]. Currently, bevacizumab, a monoclonal antibody targeting vascular endothelial growth factor, remains the only Food and Drug Administration approved drug as a single-agent for the treatment of patients with recurrent or progressive GBMs. Despite the encouraging beneficial effects seen in a number of clinical trials, this anti-angiogenic therapy has not produced the therapeutic responses initially envisioned [6,7]. Emerging evidence from both the clinical and laboratory studies suggests that GBM rapidly adapts to anti-vascular endothelial growth factor therapy leading to rapid tumor progression, and the patients who progress following bevacizumab treatment, poorly respond to bevacizumab-based combinations [7,8]. Thus, considering the overall failure of these approaches in the treatment of GBM, there is an urgent need for more effective therapies to achieve improved outcomes in newly diagnosed and recurrent GBM patients.

Chemotherapy is of limited use as treatment for GBM either because of acute systemic toxicities, or poor penetration of the blood-brain barrier (BBB) [9,10]. Doxorubicin (Doxo), an anthracycline antibiotic, is a highly effective therapeutic agent for the treatment of many malignant tumors, however, its dose-related systemic toxicity and lack of penetration through the BBB limit its use in the treatment of intracranial tumors [11,12]. Several different formulations of doxorubicin have been developed, including pegylated liposomal doxorubicin (Doxil) [13–15]. However, none of these agents showed activity in preclinical studies or in phase I/II clinical trials as a treatment option for brain tumors.

Aldoxorubicin (Aldoxo; CytRx Corporation), formerly known as INNO-206, is a (6-maleimidocaproyl) hydrazone conjugate of doxorubicin (Doxo), which binds rapidly and selectively to the Cysteine-34 position of circulating serum albumin after intravenous administration, and releases Doxo selectively at tumor sites because of the low pH of the tumor environment [16]. Once released intracellularly, the Doxo intercalates DNA, inhibits DNA synthesis, and induces apoptosis. Preclinically, Aldoxo has demonstrated superior anti-tumor activity relative to Doxo in tumor xenograft mouse models for breast, ovarian, pancreatic, and lung cancers [17,18]. More recently, Aldoxo has demonstrated activity against multiple myeloma cells *in vitro* and *in vivo*, as well as enhancement of the anti-tumor effect of bortezomib (velcade), a drug approved for the treatment of relapsed multiple myeloma [19]. A phase 1 study of Aldoxo demonstrated its safety and favorable clinical responses in a variety of tumor types [20]. Because of these encouraging results we examined the preclinical efficacy of Aldoxo against GBM using a mouse intracranial GBM model in which progression of the tumor growth, CNS invasion, as well as penetration of the drug into tumor and normal brain could be studied in a quantitative fashion.

Materials and Methods

Intracranial Implantation of U87-luc Glioma Cells in Mice

A human GBM U87MG subline, U87-luc stably integrated with a luciferase reporter gene [21], kindly provided by Dr. Daniel A. Vallera [22], was used for establishing intracranial xenograft GBM tumors. Female athymic nude mice, 6 to 8 weeks of age (Harlan Laboratories), were anesthetized with a ketamine/xylazine cocktail solution. Animals were secured in a Harvard Apparatus stereotaxic head frame, a 1-cm midline scalp incision was made, and 5×10^5 cells in 5 µl serum-free DMEM were injected into the left striatum (coordinates: 2.5 mm lateral and 0.5 mm posterior to the bregma) through a burr hole in the skull using a 10-µl Hamilton syringe to deliver tumor cells to a 3.5-mm intraparenchymal depth. The burr hole in the skull was sealed with bone wax and the incision closed using Dermabond. Tumor growth was monitored and measured via bioluminescence imaging *in vivo*. All experiments were performed in accordance with the Institutional Animal Care and Use Committee guidelines.

Drug Treatment of Mice

Aldoxorubicin (Aldoxo; CytRx Corporation, Los Angeles, CA) and Doxo (Sigma-Aldrich, cat # 44583) were prepared on each day of injection in sterile vehicle (10 mM of sodium phosphate, 5% of D-(+)-glucose, pH 6.4) at a concentration of 10 mg/3 ml and 3 mg/3 ml, respectively. The dosing formulations were stored at 4°C before injection and were injected within 1 hour of formulation. Both drugs were administered intravenously in a volume of 0.15 ml to achieve 75% of the maximum tolerated Aldoxo dose of 32 mg/kg per injection and Doxo dose of 8 mg/kg per injection for a 20-g mouse. The study consisted of 8 vehicle-treated control mice (group C), 8 Doxo-treated mice (group D), and 8 Aldoxo-treated mice (group A). Treatment was initiated 12 days after intracranial implantation of GBM cells. Vehicle or Aldoxo was administered for a total of six injections (i.e., 12, 19, 26, 42, 50, and 56 days after cell implantation). Doxo was administered intravenously for a total of only two injections (i.e., 12 and 19 days after cell implantation), because seven of the eight mice in this group died before the third injection.

In Vivo Imaging of Intracranial Tumors

Intracranial tumor growth was quantified by biophotonic imaging using a Xenogen IVIS 200 system (Xenogen, Palo Alto, CA). Mice were administered a 100-µl intraperitoneal injection of 30-mg/ml D-luciferin (PerkinElmer) suspended in DPBS (Gibco) 10 minutes before imaging as a substrate for the luciferase enzyme. Prior to imaging, anesthesia was induced with isoflurane gas by placing mice in the chamber of an XGI-8 vaporizer, and sustained by inhalation via nose cones inside the imaging chamber. Images were captured and quantified with Living Image 4.1 software based on equivalent regions of interest over the head. Image intensities were expressed as photons per sec/cm^2 per steradian.

Neurotoxicity and Morbidity Assessments

Neurotoxicity was assessed by signs of motor disturbances and/or imbalance, and morbidity by rapid weight loss, impaired mobility, decreased food intake, and signs of lethargy.

High-Performance Liquid Chromatography (HPLC) System and Conditions

The HPLC system used was an Agilent 1100 Series (Wilmington, DE) equipped with a scanning fluorescent detector with excitation

and emission wavelengths set at 480 and 560 nm, respectively. Agilent Chemstation software was used for data acquisition. Separation was achieved on a Waters Spherisorb ODS2 column (4 mm × 250 mm, 5 μm) fitted with a guard cartridge (BDS-Hypersil-C18, 5 μM). Elution was performed with mobile phase comprised of 65% 50 mM monosodium phosphate, pH 2.2, and 35% acetonitrile. A constant flow rate of 1.25 ml/min was used for the separation. The column was set to 28°C and the injection volume was 25 μl.

Doxo, Aldoxo, and the internal standard daunorubicin (Sigma-Aldrich, cat # 30450) demonstrated average retention times of 4.06, 4.39, and 6.52 min, respectively, and were sufficiently resolved under the applied assay conditions. In the organ samples analyzed, Aldoxo eluted with the retention time of Doxo. No interfering peaks were observed under the chromatography conditions used.

Sample preparation. For quantification of Aldoxo in brain and brain tumors, Aldoxo, 24 mg/kg per injection (18 mg/kg Doxo equivalents; 75% of the maximum tolerated dose (MTD)), was administered to intracranial GBM tumor-bearing and non-tumor-bearing mice via tail vein injection. After 6 and 24 hours of Aldoxo injection, mice (n = 3 per time point) were euthanized by CO_2 inhalation, brains were harvested and tumors were resected. The harvested brain and tumor tissues were stored at − 80°C until analysis.

Frozen samples were thawed at room temperature and homogenized in sterile saline using a PowerGen Model 125 homogenizer (Fisher Scientific) to obtain final tissue concentrations (w/v) of 150 mg/ml. Perchloric acid (35%, v/v) was then added to a 20-μl aliquot followed by 25 μl of mobile phase. The samples were vortexed followed by centrifugation at 10,000 × *g* for 10 minutes and 25 μl of the supernatant was applied to the HPLC column. In the tissue samples analyzed, Aldoxo eluted with the retention time of Doxo.

Immunohistofluorescence

For histologic analysis, brain tissues from control and drug-treated tumor-bearing mice were harvested, snap frozen in optimal cutting temperature embedding medium and stored at − 80°C. Cryostat sections were placed on slides and fixed in zinc-buffered formalin. Slides were blocked with 5% goat serum in 1% BSA followed by overnight incubation with primary antibodies against CD31 (102402, Biolegend, San Diego, CA), Ki-67 (ab156956, Abcam, Cambridge, MA), Vimentin (ab92547, Abcam), and cleaved-Caspase-3 (CP229B, Biocare Medical). Slides were then incubated with secondary antibodies conjugated to Alexa Fluor 488 or 635, washed and treated with 4′,6-diamidino-2-phenylindole as a nuclear counterstain. The detection fluorophores used were limited to those around the inherent fluorescence spectra of Doxo (λ_{ex} = 480 nm, λ_{em} = 550 to 590 nm) [23] to avoid bleed-through and enable co-detection of the drug with respect to certain antigens. Epifluorescence photomicrographs were captured at 100 × and 400 × magnification using an FV1000 confocal microscope (Olympus of America, Center Valley, PA) equipped with multi-Argon, 405, 559, and 635 diodes. Quantitative analysis was performed with Slidebook 5 software (Intelligent Imaging Innovations, Denver, CO).

Aldoxo/Doxo Detection in Brain Tumors

Tumor-bearing mice were given intravenous injections of Aldoxo or Doxo as described above. Mice were euthanized 24 hours following the last injection. Brains were harvested and imaged using an MVX10 stereomicroscope (Olympus of America) equipped for brightfield and epifluorescence with filters encompassing Doxo-specific wavelengths to visualize drug accumulation.

Statistical Analysis

Analyses were carried out in SAS 9.3 (SAS Institute, Inc., Cary, NC). Radiance data for experimental groups (Figure 4*A*) were assessed by the Kruskal-Wallis test followed by Dunn's multiple comparisons. Kaplan-Meier estimates of group survival functions were obtained and pairwise comparisons were performed by exact log-rank tests [24]. The median survival time for each experimental group and its 95% confidence interval was also estimated. $P \le 0.05$ was considered statistically significant.

Results

Aldoxo Inhibits Intracranial Growth of GBM Tumors

To evaluate anti-GBM efficacy of Aldoxo, we injected 5×10^5 U87-luc cells (human GBM U87MG cells stably expressing luciferase reporter) intracranially into immunodeficient mice, and the tumor cells were allowed to grow for 12 days without treatment. Subsequently, all tumor-bearing mice were randomly divided in to three groups (n = 8) and each mouse received a series of intravenous injections: Group A: Aldoxo [24 mg/kg per injection, 3/4 MTD]; Group D: Doxo [6 mg/kg per injection (3/4 MTD)]; and Group C: vehicle, (10 mM sodium phosphate, 5% D-(+)-glucose, pH 6.4); the injections were repeated once a week for the duration of the experiment (up to 60 days). Tumor growth was monitored weekly using *in vivo* quantitative bioluminescence (Xenogen IVIS-200). The results in Figure 1 show individual bioluminescence images of the brain tumors from the three experimental groups (A, Aldoxo; D, Doxo; and C, vehicle), which were taken at 8, 16, 22, 27, 34, and 41 days following the intracranial implantation of U87-luc cells. At the 8-day time point, most of the mice showed development of small brain tumors; three mice (A17, D12, and C4) exhibited tumors that were relatively larger, and two mice (D16 and C7) had no detectable tumors. At the 16-day time point, all 24 mice had intracranial tumors which varied in size, although Group A appeared to exhibit smaller tumors than the other groups. At the 22-day time point, tumor growth in mice from Groups C and D appeared to be highly aggressive, with 3 mice dead in group C and 2 in group D as a result of large brain tumors. All mice in group A were alive and demonstrated significantly smaller tumors compared with the tumors in groups C and D. At the 27-day time point, 13 mice from groups C and D were dead and the remaining three (C1, C3, D14) had very large tumors. All mice in group A remained alive and had tumors which were significantly smaller in comparison to tumors in the control and Doxo-treated mice. By the 34-day time point, all mice from groups D and C were deceased, whereas all mice in group A were still alive with minimal tumor progression. At Day 41, one mouse from Group A died, while the remaining mice did demonstrate increased but slow tumor progression.

Quantification of changes in the tumor size over time, are shown in Figure 2. The tumor sizes were calculated according to luminescence intensity expressed as radiance (photons per sec/cm^2 per steradian) × 10^7. During the first 16 days following the initial injection of the drugs all intracranial tumors increased in size without any significant differences between the control, Doxo-treated, and Aldoxo-treated mice. The first significant difference was observed at day 22, when the tumors in Aldoxo-treated mice were ~ 4-fold (P = 0.0054) and ~ 3-fold (P = 0.052) smaller when compared to the tumors in the control and Doxo-treated mice,

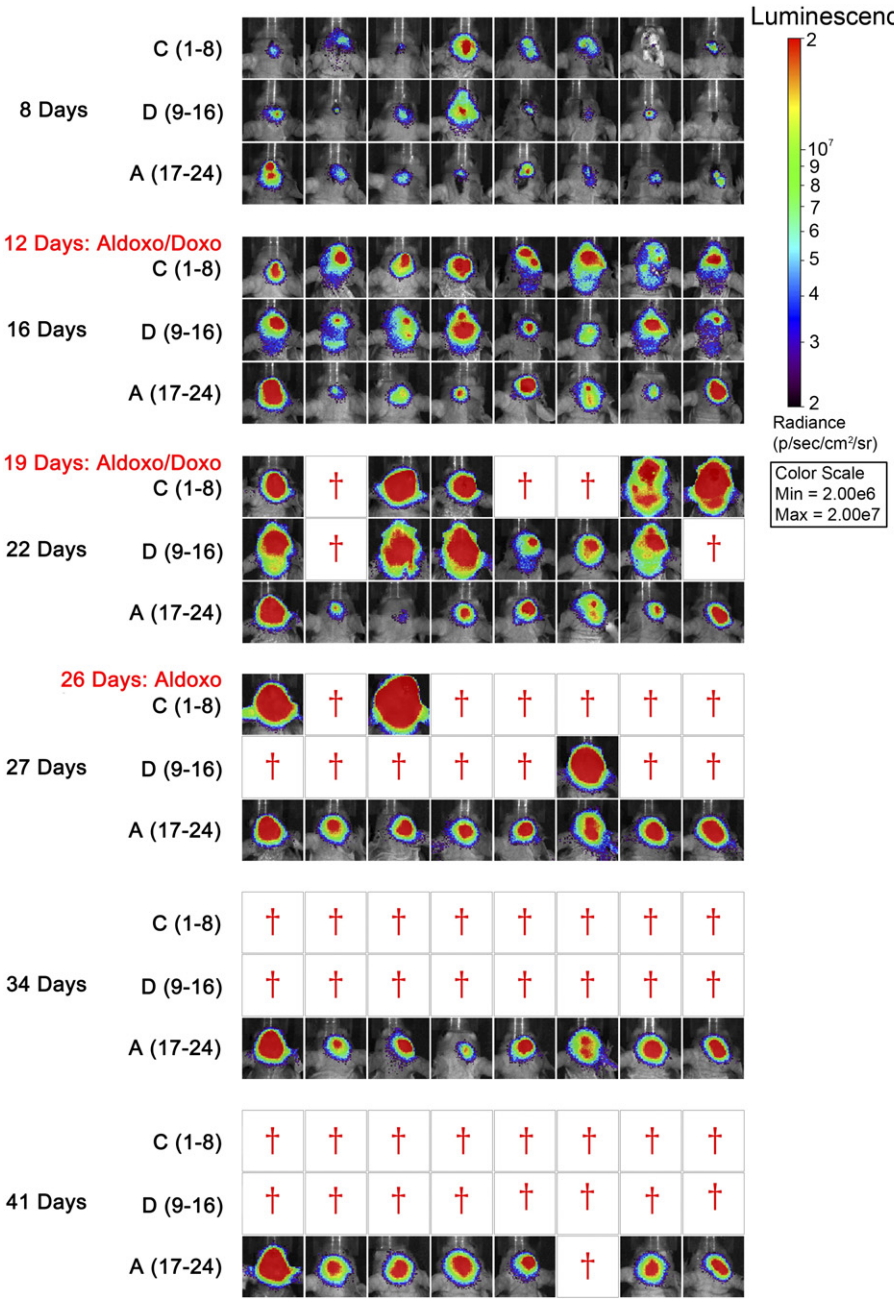

Figure 1. Biophotonic measurement of orthotopic GBM xenografts in vivo provide evidence that Aldoxo has a significant anti-tumor effect when compared to Doxo-treated or untreated tumor-bearing mice. Female athymic nude mice were intracranially infused with 5×10^5 firefly luciferase-labeled U87MG (U87-luc) glioma cell. Mice were divided into control (C), Doxo (D)-, and Aldoxo (A)-treated groups with n = 8 per group. After 12 days, groups C (1-8), D (9-16), and A (17-24) were injected with drug vehicle, 120 μg per injection of Doxo (based on a 20 g body weight), or 480 μg per injection of Aldoxo, respectively. Injection with Doxo was repeated after 19 days and with Aldoxo after 19 and 26 days. Bioluminescence imaging of brain tumors was performed after 8, 16, 22, 27, 34, and 41 days of tumor cell implantation and is shown as a function of total radiance in photons per sec/cm^2 per steradian. Tumor burden is demonstrated by a colorimetric scale where red represents the highest range of radiance values which translates to tumor burden. Red daggers indicate death of the animal.

respectively. This difference further increased, and reached 10-fold for control animals (P = 0.035) and 8-fold for Doxo-treated animals (p = 0.37, not significant because of the large standard deviation) by day 27. At this time point, all the eight mice in the Aldoxo group were still alive, whereas two mice in the Doxo group and one mouse in the control group died during the imaging procedure. Tumors in the dead mice were measured postmortem. After 34 days, when all Doxo-treated and vehicle-treated mice were deceased, the remaining Aldoxo-treated mice demonstrated a slow increase in tumor size. The experiment was terminated at the day 62, when five Aldoxo mice were still alive but started to appear moribund.

Effects of Aldoxo Treatment on the Survival Time of Mice Bearing Intracranial U87-luc GBM Tumors

Whereas all mice in the vehicle-treated (control) and Doxo groups died within 30 days of tumor cell implantation, five of the Aldoxo-

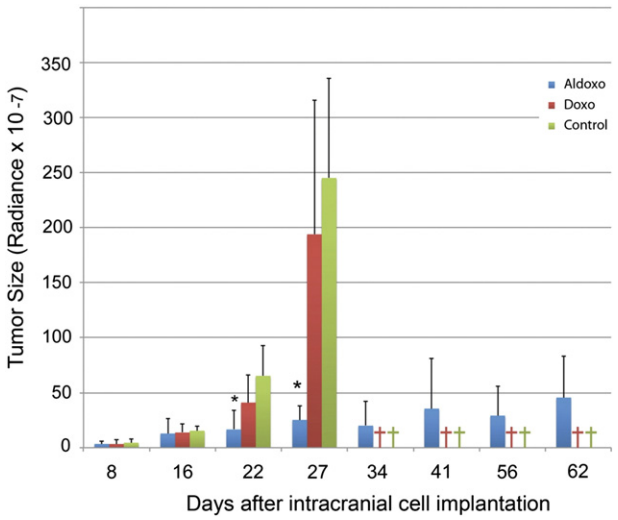

Figure 2. Tumor-bearing mice treated with Aldoxo display decreased tumorigenesis and increased survival when compared to untreated and Doxo-treated groups. Radiance values from identical regions of interest of untreated, Doxo-, and Aldoxo-treated tumor-bearing mice from Figure 1 were averaged and compared at selected time points following intracranial implantation of U87-luc cells. *Aldoxo treatment vs. control after 22 days ($P = 0.005$) and 28 days ($P = 0.035$) (t test).

treated mice survived for 62 days with three mice unable to recover from isoflurane anesthesia used during imaging and two euthanized because of the moribund condition. The results shown in Figure 3, demonstrate a highly significant improvement in the survival time in Aldoxo-treated mice as compared with control or Doxo-treated mice ($P = 0.0002$ for each comparison). The median survival times (95% confidence interval) of mice in the control, Doxo-treated and Aldoxo-treated groups were 25.5 (20, 28), 26.0 (19, 27) and 62.0 (34, 62) days, respectively. Thus, intravenous Aldoxo treatment increased the median survival time by 36 days, which represents an extension in the life span of mice bearing

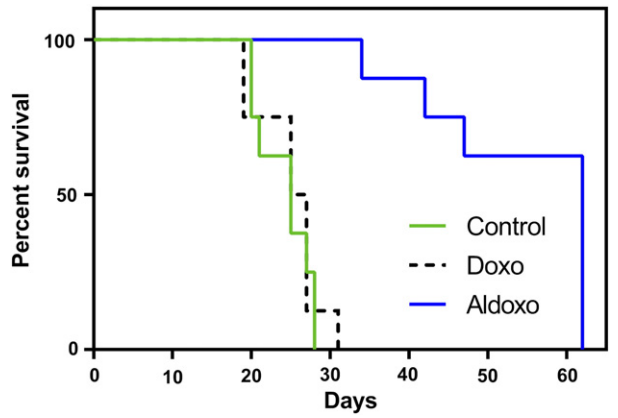

Figure 3. Tumor bearing mice experience significantly longer survival following treatment with Aldoxo. Kaplan-Meier curves are compared between mice bearing U87-luc tumors treated with vehicle, Doxo, and Aldoxo. Doxo and Aldoxo were administered at 75% MTD as described in Methods. Statistical analysis of n = 8 per group reveals significantly longer survival ($P = 0.0002$) of mice treated with Aldoxo when compared against either vehicle or Doxo-treated mice.

intracranial GBM by 138% [(36/26)*100]. The average difference in the median survival time between the control and Doxo-treated mice was only 0.5 day ($P = 0.715$).

Accumulation of Aldoxo and Doxo in the Brain and Brain Tumor Tissue Following Intravenous Drug Delivery

HPLC was used to detect Aldoxo content (elutes with the retention time of Doxo) in the intact brain and the brain tumor tissue following intravenous injection of Aldoxo (Figure 4*A*). At the 6-hour time point following a single tail-vain delivery of Aldoxo (24 mg/kg per injection), the average concentration of the drug in the brain tumor tissue was over four-fold greater than in the adjacent intact brain tissue. At the 24-hour time point, the concentration of Aldoxo declined significantly in both brain and brain tumor tissue, however it was still at least two-fold higher in the tumor. Interestingly, Aldoxo was undetectable in the brain tissues of non-tumor-bearing mice, suggesting that Aldoxo detection in the brain tissues of tumor-bearing mice may be the result of disrupted blood brain barrier or other structural alterations in the brain tissue surrounding the tumor.

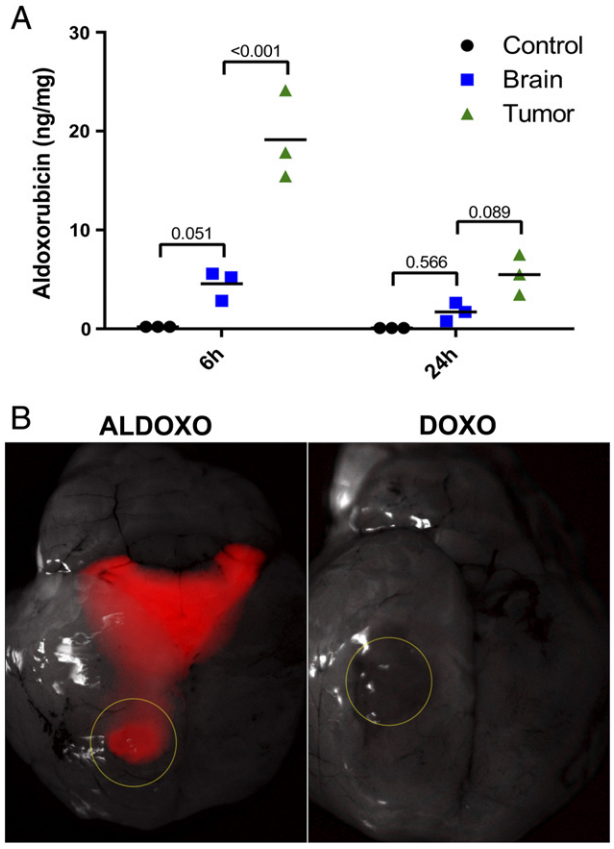

Figure 4. High accumulation of Doxo is specific to orthotopic GBM xenograft tissue but only during treatment with Aldoxo. (A) Tumor and surrounding brain tissue from Aldoxo-injected mice were selectively harvested at 6 and 24 hours post-treatment and processed for HPLC. Data represent average values of Aldoxo (elutes with the retention time of Doxo) concentration (ng/mg) with statistical significance of differences between Aldoxo-treated non tumor-bearing (control), and tumor-bearing mice. (B) Whole brains harvested from Aldoxo- and Doxo-treated mice were photomicrographed by epifluorescence stereomicroscopy to visualize the extent of Doxo-specific fluorescence around the stereotactic infusion site of U87MG cells (yellow circle).

We confirmed our HPLC results by taking advantage of the natural fluorescence of Doxo (excitation 480 nm, emission 500 to 590 nm). The brains were harvested 24 hours following the tail-vein injection of Aldoxo and Doxo, and were imaged using an epifluorescence stereomicroscope. As shown in Figure 4*B*, the Aldoxo-treated mice demonstrated a strong red fluorescence at the injection site of the tumor cells (yellow circle), and the expansion of the fluorescence towards the occipital region of the brain, which in this particular case corresponded to the area of the tumor growth. In contrast, we did not observe any red fluorescence in the brain or in the brain tumor tissues of Doxo-treated mice, further supporting the unique ability of Aldoxo to reach intracranial GBM tissue, and having only a minimal effect on the surrounding brain tissue. To evaluate if Doxo-specific fluorescence seen macroscopically (Figure 4*B*), results also in the selective incorporation of the drug into the tumor cells, we examined cryosections of the brain tissue from the control, Doxo-treated and Aldoxo-treated mice. The results in Figure 5 demonstrate an abundant accumulation of Doxo (red fluorescence) in the area occupied by the brain tumor from the Aldoxo-treated mice (panels C-C′ and D). The tumor cells from the control and Doxo-treated mice were completely negative (panels A-A′ and B-B′). The image depicted in panel D is a higher magnification of the margin between the tumor and unaffected brain tissue (rectangle from panel C), which clearly indicates that

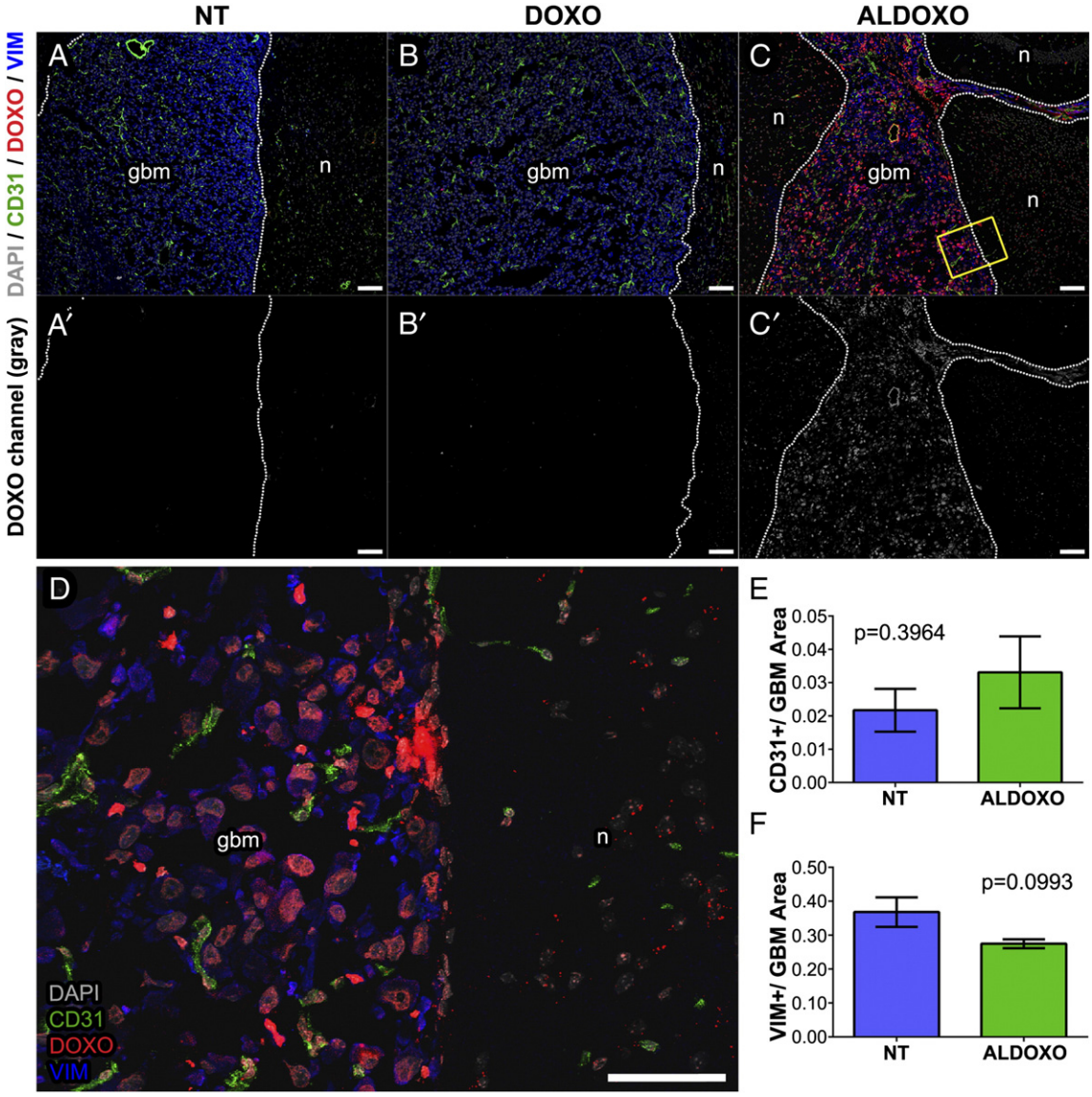

Figure 5. Doxo fluorescence is highly localized to cells in orthotopic U87-luc xenografts but only in tumor-bearing mice treated with Aldoxo. (A-C) Frozen sections of brain tissue housing tumor xenografts were co-immunostained against Vimentin (VIM, blue) and CD31 (green) to label tumor-specific area and vasculature, respectively, and counterstained with nuclear 4′,6-diamidino-2-phenylindole for confocal imaging at 100× magnification (100-μm bar). The innate red fluorescence of Doxo was also co-detected with respect to these two markers and this channel was extracted in (A′-C′) grayscale for better demonstration of the localization of Doxo between samples and within glioblastoma (gbm) and normal (n) tissue. (D) Subsampling of a region of interest from the Aldoxo treated tumor in C (yellow rectangle) at 400× shows localization of Doxo to the cytosol and nuclei of VIM+ U87-luc cells with minimal expression in adjacent brain tissue (50-μm bar). (E, F) The effect of Aldoxo on tumor content and vascular area was assessed by quantitation of CD31+ and VIM+ cells over total tumor area.

the majority of the tumor cells are strongly positive for the intracellular Doxo-specific red fluorescence.

In addition, the same sections were immunolabeled using anti-CD31 (green) and anti-Vimentin (blue) antibodies to evaluate possible effects of the Aldoxo-treatment on the tumor vascularization and the expression of vimentin, a pro-invasive intermediate filament protein, respectively. It is noteworthy that, we did not observe the expected reduction in the vascularization (CD31-positive immunolabeling) of the tumors from Aldoxo-treated mice that were strongly positive for Doxo red fluorescence. Although not significant (P = 0.3964), the CD31 immuno-positivity was actually higher in these tumors (panel E). Aldoxo-treatment was associated with a slight reduction in the number of vimentin-positive tumor cells, although not significant (P = 0.099) (panel F).

Effect of Aldoxorubicin Treatment on Tumor Cell Proliferation and Apoptosis

To assess the effects of the Aldoxo-treatment on tumor cell proliferation and apoptosis, cryosections of the intracranial tumor tissue were co-immunolabeled with anti-Ki67 and anti-cleaved Caspase-3 antibodies, respectively. The results in Figure 6, *A* and *B* demonstrate that in comparison to the control vehicle-treated mice, the Aldoxo-treatment reduced the number of Ki-67 positive tumor cells over 3-fold (P = 0.018) *per* tumor area (Figure 6*C*). Co-detection of apoptosis with anti-cleaved caspase-3 within the same section demonstrated a highly significant increase (P = 0.0005) in pro-apoptotic signaling in the Aldoxo-treated mice compared to tumors from the control mice (Figure 6, *D–F*). This observation indicates that cells within the tumor appear to undergo apoptosis following intravenous delivery of Aldoxo. Importantly, we did not observe programmed cell death in the brain areas adjacent to the tumor tissue (not shown), supporting that Aldoxo treatment does not have an adverse effect on the normal cells even at the tumor margins.

Discussion

GBM is the most common and lethal CNS malignancy, accounting for nearly 60% of the primary brain tumors [1]. Although aggressive surgery combined with radiation and temozolomide chemotherapy prolong survival of GBM patients, these tumors frequently acquire chemoresistance. A number of therapeutic strategies have been evaluated over the years in patients with recurrent or progressive disease, but no consistent survival benefit has been observed. Thus, the development of new more effective treatments to achieve durable outcomes remains a major therapeutic challenge.

To be effective, a cytotoxic drug must reach tumor cells at therapeutic concentrations. Although, Doxo is a highly efficient chemotherapeutic agent for the treatment of a wide variety of systemic cancers, its clinical application is limited in the treatment of brain tumors because of its inability to penetrate the BBB [12,25]. Thus, in recent years, efforts have been made to develop novel compositions of Doxo that exhibit reduced toxicity and improved therapeutic efficacy (cf. pegylated liposomal Doxo, Doxil) [13–15]. Although these new approaches improve the half-life of the drug and reduce some of its systemic toxicities they do not overcome the problem of effective penetration and release of the active drug in brain tumor tissue. In the present study, we analyzed the antitumor activity of a conjugated Doxo that binds covalently to circulating albumin (Aldoxo) and compared its efficacy to free Doxo in immunodeficient mice bearing intracranial human U87-luc GBM tumors. Aldoxo is a novel drug-conjugate of Doxo in which Doxo is derivatized at its C-

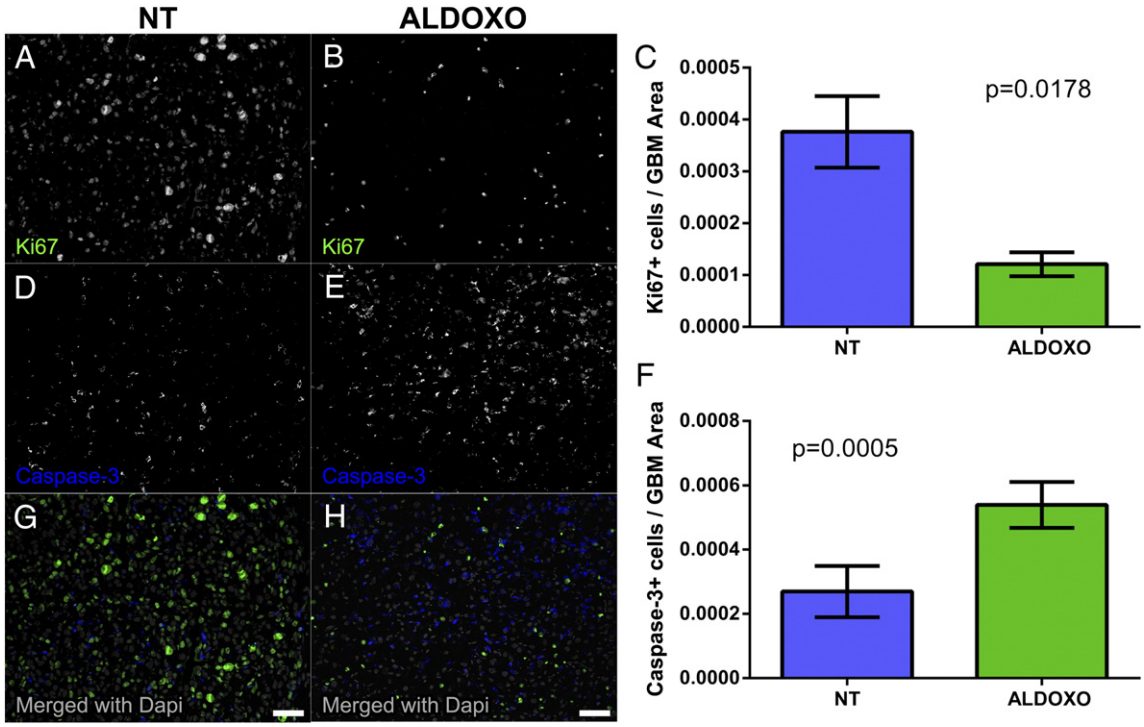

Figure 6. Treatment with Aldoxo decreases the proliferation index of tumor cells and triggers increased apoptosis in xenografts. Immunodetection and quantitation of the proliferation marker (A-C, G, H) Ki-67 and effector of apoptosis (D-F, G, H) reveal significant differences in the survival dynamics of non-treated (NT) and Aldoxo-treated tumor tissue. Calibration bar = 50 μm.

13 keto-position with a thiol-binding spacer molecule, 6-maleimidocaproic acid hydrazide. This modification allows Aldoxo to bind selectively to the cysteine-34 residue of the circulating serum albumin after intravenous administration [16,17] with release of Doxo either extracellularly in the slightly acidic environment present at the tumor site or intracellularly in the acidic endosomal or lysosomal compartments of the tumor cells [17,26].

Our results demonstrate that in comparison to free Doxo, which was ineffective against intracranial GBM, Aldoxo had a pronounced antitumor activity in our animal model. Aldoxo-treated mice demonstrate significantly slower growth of the tumor and prolonged the survival time of the tumor bearing mice by more than 100% as compared to vehicle-treated and Doxo-treated mice. Importantly, Aldoxo-treated mice demonstrate accumulation of Aldoxo exclusively at the brain tumor sites suggesting its effective penetration through the blood brain-tumor barrier but not through the BBB. This highly selective accumulation of Aldoxo inside the brain tumor tissue and its low penetration in to the tumor-free brain could be very important to reduce any potential neurotoxicity due to Doxo.

We further evaluated how intravenous Aldoxo-treatment affects growth and survival of the tumor cells inside the intracranial tumor tissue. Our immunohistofluorescent data using antibodies specific to the cleaved (active) form of caspase-3 and to Ki-67, a cell proliferation marker clearly demonstrated abundant apoptosis and a very low tumor cell proliferation. However, in spite of these very positive effects of Aldoxo against the tumor cells *in vivo*, we did not observe expected changes in the immunolabeling for the endothelial marker CD31 suggesting that intratumoral endothelial cell proliferation and tumor vascularization is not affected by the drug. It is possible that the environment inside the newly formed vasculature, different than tumor tissue, is not acidic enough to cleavage the drug from its carrier, albumin. Therefore, it would be important to test if a vascular-disruptive agent such as bevacizumab [27] could be used in combination with Aldoxo to target simultaneously tumor cells and neovascularization in GBM.

The median lethal dose of Aldoxo in CD-1 mice is established to be >60 mg/kg (Doxo equivalents) compared with ~12 mg/kg for Doxo [28]. At 30 mg/kg (Doxo equivalents) no clinical signs other than peripheral neuropathy and motor disturbances were observed in 25% of mice which developed as a delayed toxicity following intravenous administration of the drug [28]. In our study with Aldoxo at a three-times higher dose of Doxo (18 mg/kg of Doxo-equivalents *versus* 6 mg/kg of Doxo), we did not observe any signs of neurotoxicity marked by motor disturbances and/or imbalance. Although, Aldoxo administered at 24 mg/kg (18 mg/kg Doxo-equivalents) was significantly better tolerated than Doxo at 6 mg/kg, we observed that following the last intravenous administration of Aldoxo, mice rapidly developed morbid condition which was marked by rapid weight loss, impaired mobility, decreased food intake, and signs of lethargy. We do not know yet the underlying cause of this worsening condition, but it might be a result of cumulative Aldoxo cytotoxicity, which is still much less pronounced and develops much later than with Doxo treatment.

Albumin is the most abundant plasma protein and is emerging as a versatile carrier for targeting anti-tumor drugs [28]. Solid tumors accumulate albumin due to their unique pathophysiological characteristics, such as high metabolic turnover, extensive angiogenesis, defective vascular architecture, and impaired lymphatic drainage [29,30]. While the systemic delivery of many drugs for brain tumor therapy is seriously hampered by poor drug delivery to the brain, the role of albumin in allowing penetration of drugs through the blood brain-tumor barrier is intriguing. Our results are consistent with an earlier report showing that glioblastoma patients accumulated fluorescently-labeled albumin selectively into the brain tumors and not in the normal brain [31].

In preclinical studies, Aldoxo has shown superior anticancer efficacy over Doxo in a number of murine xenograft models including renal cell carcinoma, breast carcinoma, ovarian carcinoma, and small cell lung cancer, and in an orthotopic pancreatic carcinoma model [17]. Complete remissions were achieved with Aldoxo in the renal cell carcinoma and breast carcinoma xenograft models in nude mice [16]. In a phase 1 study, Aldoxo showed a good safety profile and favorable responses in patients with solid tumors such as breast cancer, small cell lung cancer and sarcoma [20]. A potential advantage of Aldoxo is its favorable pharmacokinetics for a long circulating drug delivery system, such as a small volume of distribution, a high-plasma area under the concentration-time curve, and low clearance compared with Doxo [26]. The doses of Aldoxo used in our study (24 mg/kg per week or 18 mg/kg per week Doxo-equivalents) are clinically achievable (72 mg/m^2 Aldoxo or 54 mg/m^2 Doxo-equivalents) and are far below the well-tolerated dose of 200 mg/m^2 (150 mg/m^2 Doxo-equivalents) administered in the phase 1 clinical trial without manifestation of drug-related side effects [20]. This Doxo equivalent dose of Aldoxo is 2 to 2.5 times higher than the clinically used Doxo dose of 60 to 75 mg/m^2, which often results in moderate to severe side effects.

As a result of our preclinical study, Aldoxo has emerged as a promising clinical candidate for the treatment of brain tumors because of its selective accumulation and prolonged retention in brain tumor tissue and its potent antitumor activity against human GBM cells.

Acknowledgments

The authors thank Dr. Daniel A. Vallera (Department of Therapeutic Radiology-Radiation Oncology, University of Minnesota, Minneapolis, MN) for providing the U87-luc cell line, and Dr. Frank Culicchia (Department of Neurosurgery, LSUHSC, New Orleans, LA) for his support.

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