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Identification of Novel Agents for the Treatment of Brain Metastases of Breast Cancer

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Abstract

Background—Brain cancer from metastasized breast cancer has a high mortality rate in women. The treatment of lesions is hampered in large part by the blood-brain barrier (BBB), which prevents adequate distribution of anti-cancer compounds to brain metastases.

Method—In this study we used a novel screening method to identify candidate molecules that are well-suited to utilizing the BBB choline transporter for distribution into the brain parenchyma.

Results—From our screen we identified two compounds, Ch-1 and Ch-2 that were able to reduce the brain tumor burden in a murine mouse model of brain metastasis of breast cancer. These compounds also significantly increased the survival of mice by more than 10 days. Mechanistic studies indicated that Ch-1 is able to prevent the activation of the pro-survival mitogen-activated kinases (MAPKs) by osteoactivin (OA; Glycoprotein nonmetastatic melanoma protein B GPNMB).

Conclusion—The results from this study show that nutrient transporter virtual screening is a viable novel alternative to traditional drug screening programs to identify anti-cancer compounds for the treatment of brain cancers.

Keywords

Drug resistance; CNS; distribution; brain cancer; drug discovery; chemotherapy; ADME

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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1. INTRODUCTION

The blood-brain barrier (BBB) hinders drug delivery to the central nervous system. Passive drug permeation is limited across the BBB by the presence of specialized tight junctions formed between the vascular endothelial cells [1, 2]. Several strategies have been investigated to increase drug distribution for the treatment of brain cancers, ranging from medicinal chemistry approaches to the nanoparticle delivery systems. Another strategy is to exploit nutrient transport proteins at the BBB such that they would shuttle compounds between the blood and the brain [3].

The choline transporter (CHT) is one of these potential transport systems. It is present throughout the body, and has unique pharmacokinetic properties based on tissue distribution. Choline transport proteins present in the brain are classified into low and high affinity transport proteins. The BBB choline transporter has more moderate affinity to choline, is sodium independent and can deliver drugs without compromising endogenous choline supply to the brain [4, 5]. The BBB choline transporter has been used successfully in CNS drug delivery. For example, the cationic nicotinic antagonist N-n-octylnicotinium iodide, has about 40% brain uptake using choline transporters present at the BBB [6].

There is emerging research demonstrating the effectiveness of cationic chemotherapeutics in peripheral tumors. This class of drugs may act on the mitochondria as a metabolic poison, leading to G1 arrest and apoptosis [7]. However, because of their positive net charge, it has been assumed that these drugs will not cross the intact BBB, and therefore, little work has been done to determine if these drugs could be efficacious in the treatment of brain tumors. Recent findings demonstrate that cationic chemotherapeutics can also be delivered to brain using the BBB choline transporter. [8, 9]

Integration of absorption, metabolism, distribution, and excretion (ADME) properties early in the drug discovery process provides an alternative model for effective drug discovery and development. Based on these findings, we report the use of *in-silico* drug discovery approaches to identify cationic chemotherapeutics that meet structural requirements for transport into the brain by way of the BBB choline transporter as a method for the treatment of brain metastasis of breast cancer.

2. MATERIALS AND METHODS

2.1. Chemicals

Compounds in Fig. (**1**) were obtained from ChemBridge Corporation, USA. D-Luciferin was purchased from Caliper Life Sciences, USA. Carboxymethyl Cellulose, Sodium Lauryl Sulphate (SLS), Polyvinyl pyrrolidone and Antifoam were purchased from Sigma chemicals (St. Louis, USA).

2.2. Virtual Screening

The identification of a lead compound was completed using previously reported methods [10–12]. Because the crystal structure of the BBB choline transporter has not yet been determined, we developed a surrogate model which could be used in virtual screening. In

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short, the comparative molecular field analysis (CoMFA) study [10, 11] gave us insight into the electronic and conformational requirements for binding to the BBB CHT. We used our previously developed homology model of the choline transporter [12] which was able to recapitulate the electronic and steric properties seen in CoMFA. For virtual screening, we used ZINC databases [13], which are freely available from commercial chemical suppliers. The homology model of CHT [12] was protonated at pH 7.4 using MOE 2010.10. The databases were washed to remove any salts, and then optimized in 3D to yield a database which could be used for the docking study. The top ranked compounds from the docking study were clustered in MOE 2010.10 and we chose the most successfully-docked compound from the each cluster, based on score.

2.3. *In Vitro* Cytotoxicity Studies in MDA-MB-231 Br Cells

MDA-MB-231 Br cells were seeded at a density of 10^4 cells per well in 96 well plates. The cells were treated with varying concentrations of Ch-1 and Ch-2. Cytotoxicity was determined using MTT assay, and the IC_{50} of these compounds were determined using non-linear regression analysis.

2.4. Cell Cycle Analysis Using Flow-cytometry

MDA-MB-231 Br cells were seeded at density of 10^6 cells per well in 12 well plates and incubated for 24h to allow for cell adhesion. The cells were then treated with different concentrations of Ch-1 and Ch-2 for 48 h. After treatment, cells were trypsinized and centrifuged. The pellet was washed twice with PBS and then fixed with 70 % ice cold ethanol for 24 h. Fixed cells were then centrifuged at 300g for 5 min, and the cell pellet was washed twice with PBS to remove excess ethanol. After washing, the cells were incubated at 37°C with Propidium Iodide solution (10μg/mL) containing 0.1% Triton X-100 and 100 μg/mL DNase free RNase A in PBS. Cells were analyzed with a FACS Scanner flow cytometer (Accuri, USA).

2.5. Effect of CH-1 and CH-2 on Choline Transport

MDA-MB-231 Br cells were seeded at density of 5×10^4 cells per well in 12-well plates and incubated for 24h allow for cell adhesion. The following day, media was removed from the wells and 0.1μCi of ^{3}H-choline was added to each well. Then, varying concentrations of Ch-1 and Ch-2 (0–40 μM) were added, and cells were incubated for 20 min. After incubationl, media was aspirated, and cells were washed three times with PBS. The cells were then lysed with RIPA lysate buffer, collected and used for the determination of ^{3}H-choline concentrations. Values were normalized to mg of protein. Percentage uptake was plotted against concentration and concentration required to inhibit 50% (IC_{50}) of choline was calculated using GraphPad Prism 5 software.

2.6. Effect of Ch-1 Against OA (GPNMB) Activation of ERK1/2

MDA-MB-231 Br cells were seeded at a density of 10^6 cells per well in 12 well plates and incubated for 24h to allow for cell adhesion. Cells were treated with Ch-1 at 10 μM for 10 min. before the addition of GPNMB (R&D Systems) at 50 ng/mL. After 1 hour of treatment, the cells were washed with cold PBS and cells were trypsinized, using RIPA buffer. Protein

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lysates were run on Western Blots (Bio-Rad) and ERK 1/2 kinase activation detected using an antibody from Abnova.

2.7. *In Vivo* Efficacy of Ch-1 and Ch-2 in Preclinical Model of Brain Metastases of Breast Cancer

Brain metastases of breast cancer were developed in immune compromised NuNu female mice by injecting Luciferin transfected human MDA-MB-231 Br (MDA-MB-231 Br-Luc) cells into the left cardiac ventricle. Briefly, NuNu mice were anaesthetized with isofluorane (0.5–2%) and injected with 1.75×10^5 human MDA-MB-231 Br-Luc cells in 100μL serum free media using a stereotaxic apparatus (Stoelting Co., Wood Dale, IL, USA). Accuracy of injection was confirmed using bioluminescence imaging at 24 h post-injection. Two weeks after innoculation, animals were randomly divided into three groups of 10 animals. Animals were treated with vehicle alone, Ch-1 and Ch-2. Compounds were administered intra-peritoneally twice a week at 1 mg/Kg. The vehicle used for the preparation of Ch-1 and Ch-2 suspensions was composed of 1% Sodium Carboxymethyl Cellulose, 0.125% Sodium Lauryl Sulphate (SLS), 0.05% Polyvinyl pyrrolidone and 0.05% antifoam in de-ionized water. All formulations were prepared fresh on the day of dosing. Animals were monitored for development of neurological symptoms, at which point animals were euthanized and brains were flash frozen in isopentane and stored at −80°C for further analysis. All animal experiments were performed in accordance with approved animal use and care protocols.

2.8. Bioluminescence

Animals were monitored for tumor growth using an IVIS Imaging system. Briefly, animals were injected with D-Luciferin at a dose 150 mg/kg i.p. After 10–15 min, animals were imaged for bioluminescence utilizing IVIS™100 System and Living Image® software (Xenogen, Alameda, CA, USA) with a 5-min acquisition time. Animals were imaged for bioluminescence signal once a week. After development of neurological symptoms (within a 90 day window), animals were imaged for bioluminescent signal to confirm presence of tumor in brain and euthanized.

2.9. Histological Confirmation of Tumors Using Cresyl Violet Staining

Brain sections of 20 μm were rehydrated in cold PBS (4°C) and fixed with 4% paraformaldehyde (4°C) with subsequent washings. Cresyl violet stain (0.1% cresyl violet acetate) was added to each section for 15 mins, then rinsed with distilled water and placed in ethanol of increasing concentrations (70%, 95%, and 100%) for improved differentiation. Slides were placed in xylene for clearing, air dried, and mounted with DPX mounting medium. Cresyl violet images were used to count number of tumors present in each slice, and tumor area was determined using a masking method in Slide book 5.0. Based on the tumor area, each individual tumor was classified as small (<0.196 mm^2) medium (<0.785 mm^2 and >0.196 mm^2) and large tumors (>0.785 and <3.140 mm^2).

2.10. Apoptosis Using Click-iT Tunel Assay

Brain tissue sliced at 20um was used for the microscopic visualization of Apoptosis biomarkers. Apoptotic cells in each brain section were visualized by direct immunostaining

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using commercially available Click-iT® TUNEL (terminal deoxynucleotidyl transferase biotin-dUTP nick-end labeling) Alexa fluor 647 Imaging Assay kit (Invitrogen, USA). Briefly, brain sections were rehydrated in cold PBS (4°C) and fixed with 4% paraformaldehyde (4°C) with subsequent washings. These sections were permeabilized with 1% SDS (sodium dodecyl sulphate) and staining was carried out as per the instruction given by the manufacturer.

2.11. Statistical Analysis

Data in all experiments represent mean ± SEM. Experiments were analyzed using ANOVA followed by Bonferonni's multiple comparisons test. For the survival data, animals were verified to have BLI signal from brain 24 hrs after cardiac injection and were then randomized to the three groups. A Kaplan-Meier curve was obtained and a log-rank value was determined. A significance level of $p < 0.05$ was used for all experiments (GraphPad Prism 5 software).

3. RESULTS

3.1. Virtual Screening

The compounds retrieved from the docking study and incorporated into the homology model of CHT that were chosen for this study are shown in Fig. (**1**). The top ranked compounds from the docking study were clustered in MOE 2010.10 thereby increasing the chances of identifying novel chemotypes. In each cluster, we chose the compound with the best-docked score. As can be seen from Fig. (**1**), we identified 16 compounds which were then acquired from the suppliers (Chembridge, www.hits2lead.com). The top ranked compounds Ch-1 and Ch-2 were investigated for *in vitro* and *in vivo* activity.

3.2. Effect of Ch-1 and Ch-2 on Choline Transport

The binding affinities of Ch-1 and Ch-2 to choline transporters were studied using ^{3}H-Choline in MDA-MB-231 Br-Luc cells. Ch-1 and Ch-2 compounds showed inhibition of 50% of ^{3}H-Choline binding to cells at 8.52±0.25 μM and 9.17±0.15 μM respectively (Fig. **2A** and **2B**). This indicates that both compounds have high affinity (<10μM) for choline transporters in MDA-MB-231Br-Luc cells. Ch-1 was showed a greater affinity, however, in comparison to Ch-2 ($p<0.05$).

3.3. *In Vitro* Cytotoxicity, Apoptosis and Cell Cycle Analysis in MDA-MB-231 Br Cells

Compounds were screened for anticancer activity in MDA-MB-231Br-Luc cells using MTT assay. Both of these compounds showed anticancer activity in MDA-MB-231Br-Luc cells after the treatment for 48h. IC_{50} values of Ch-1 and Ch-2 were found to be 13.5±1.2μM and 22.9±1.4μM respectively (Fig. **3A** and **3B**). Ch-1 had a more potent anticancer effect as compared to Ch-2 ($p<0.05$). In-vitro apoptosis in MDA-MB-231Br-Luc cells was studied after treatment of cells with test compound for 48h (Fig. **3C**). Microscopic data was analyzed using Slide Book 5.0 software and 70% of cells had undergone apoptosis after treatment with Ch-1, whereas 30% of cells had undergone apoptosis after treatment with Ch-2. These results indicate that Ch-1 had more apoptotic activity in comparison to Ch-2 ($p<0.05$). Cell cycle arrest due to the activity of these compounds was studied using flow

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cytometry. Briefly, cells were treated with equimolar concentrations of either Ch-1 or Ch-2 and cellular DNA content was measured after staining the cells with propidium iodide. Both these compounds showed cell cycle arrest in G_0/G_1 phase (Fig. **3D**). Both Ch-1 and Ch-2 compounds caused significant (n=3, $p<0.05$) cell cycle arrest at G_0/G_1 phase (76.0±4.9% and 58.2 ± 7.1% of cells respectively) as compared to control (46.5± 5.1%). Further, after treatment with Ch-1 and Ch-2 there was a similar degree of apoptosis as evidenced by Click-iT® TUNEL staining.

3.4. *In Vivo* Efficacy in Preclinical Model of Brain Metastases of Breast Cancer

Antitumor activity of Ch-1 and Ch-2 was studied in a preclinical model of brain metastasis of breast cancer. Immediately after injection of MDA-MB-231Br-Luc cells, animals were imaged for bioluminescence to verify the presence of metastatic cells in brain region. Animals were randomized into three groups after verification. Each group of animals received either vehicle or Ch-1 or Ch-2, administered through intraperitoneal injection (1mg/Kg) twice a week. Tumor growth was significantly decreased with Ch-1 as compared with control and Ch-2 (Fig. **4A** and **4B**). Animals receiving Ch-1 demonstrated significantly ($p<0.05$) longer overall survival compared to control and Ch-2 group. The median survival time (MST) was 35, 36 and 45 days for control, Ch-1 and Ch-2 respectively (Fig. **4C**).

Once the animals were sacrificed, the brains were collected and sliced to 20μm sections. Cresyl violet images of the 20μm brain sections were used for quantification of the number of tumors in each brain. Percentage of metastatic tumors were significantly decreased to 40.6±2.7% and 56.2±3.8% with both Ch-1 and Ch-2 respectively compared to control ($p<0.05$, Fig. **4A**). But percentage of metastatic tumors in Ch-1 group was significantly less than Ch-2 ($p<0.05$). These metastatic tumors were classified into small (<0.2 mm^2), medium (>0.2 and <0.8 mm^2) and large (> 0.8 mm^2) tumors based on the area of tumor region. Tumor area was determined by masking method using Slide book 5.0. There was no significant difference observed in the percentage of small and medium tumors among the three groups, but a statistically significant difference in the percentage of large tumors was observed in Ch-1 group compared with control ($p<0.05$).

Tumor-bearing brain slices of Ch-1 and Ch-2 group animals showed apoptosis whereas control group animals did not show any apoptosis. Animals treated with Ch-1 demonstrated a significantly ($p<0.05$) greater percentage of apoptotic cells (66.8±4.2%) compared to Ch-2 (37.3±5.4%). Microscopic data was analyzed using Slide Book 5.0 software, and 70% of cells had undergone apoptosis after treatment with Ch-1, whereas 20% of cells were showed apoptosis after treatment with Ch-2. These results indicate that Ch-1 possesses significantly greater apoptotic activity than to Ch-2 ($p<0.05$).

3.5. Ch-1 Prevents ERK1/2 Activation by OA (GPNMB)

Data from Fig. (**5A**) which showed a reduction in the number of metastatic tumors led us to evaluate the possible link of the activity of Ch-1 to the OA (GPNMB) activation of pro-survival kinases. The activity of Ch-1 against the metastatic factor OA (GPNMB) was studied *in vitro*. As can be seen from (Fig. **5**), Ch-1 was able to prevent the activation of ERK1/2 kinases by OA (GPNMB).

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4. DISCUSSION

In this report we describe, for the first time, the use of membrane transporter modeling in the identification of novel anti-cancer compounds for the treatment of brain metastasis of breast cancer. Two compounds where identified which were able to attenuate the development of brain tumors and result in an increase in life expectancy in an *in vivo* tumor cancer model.

The BBB is rich in nutrient transporters, which allow the shuttling of nutrient factors to the brain, including the charged molecule choline. Choline is used in biochemical pathways as precursor to the neurotransmitter acetylcholine. Under physiological conditions, these transporters are capable of transporting other compounds, like drugs, without affecting nutrient uptake due to the relatively large Km or saturation kinetics [5]. These nutrient transporters, therefore, can be used as drug delivery vectors to increase uptake of compounds to the brain [5].

Most drug discovery programs start with a screening program to target a specific therapeutic pathway, such as a kinase [14]. Since a large number of compounds fail in clinical trials due to poor distribution and pharmacokinetic (DMPK) properties, we initiated this study by focusing on a new approach, which was to screen compounds for affinity to the BBB choline transporter. We utilized a virtual screen molecular modeling system developed by our lab [8]. This method allowed us to identify several compounds that have increased probability of serving as substrate for the BBB choline transporter, thereby enriching our sample sets with compounds that already are suited for therapeutically relevant *in vivo* DMPK properties to the brain tumors.

We identified two compounds from our screening that were promising candidates for *in vivo* testing. Compound Ch-1 was specifically effective in the treatment of brain tumors originating from a metastatic breast cancer model [15]. In this model, we observed that Ch-1 extended survival ~ 10 days. This increase in life span was accompanied by a statistically significant reduction in the tumor burden. Because the invasive and migratory character of breast cancer cells is in part mediated by OA (GPNM) [16], we further analyzed whether the reduction in tumor spread/ number of metastatic tumors was based on a possible mechanism of Ch-1 via the OA/GPNB mediated pathway. In cells treated with compound Ch-1 and OA, a reduction in the activation of the prosurvival kinase ERK1/2 was seen, suggesting that, in part, the lower number of metastatic tumors in the mice is due prevention of metastatic cell survival. (Fig. 6) [17].

CONCLUSION

In this study we utilized a novel virtual screening method of BBB nutrient transporters to identify possible anti-cancer compounds which are active against brain metastasis of breast cancer. We identified two compounds which are able to increase survival and reduce tumor burden. This method has the potential of increasing the ability of drug discovery programs to more quickly identify compounds which have the desired properties of BBB penetration behavior and reinvigorate discovery pipelines.

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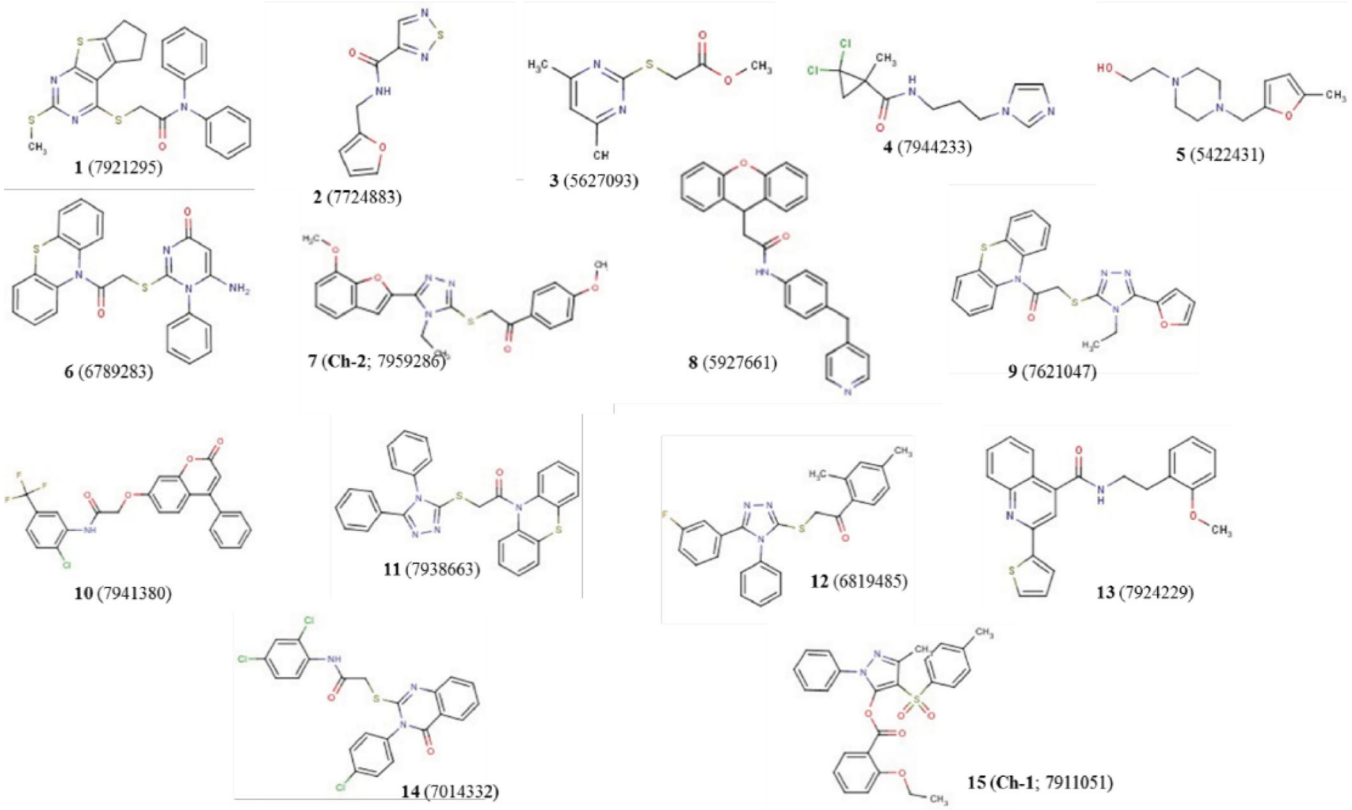

Fig. (1).
Structures of compounds identified from virtual screening study.

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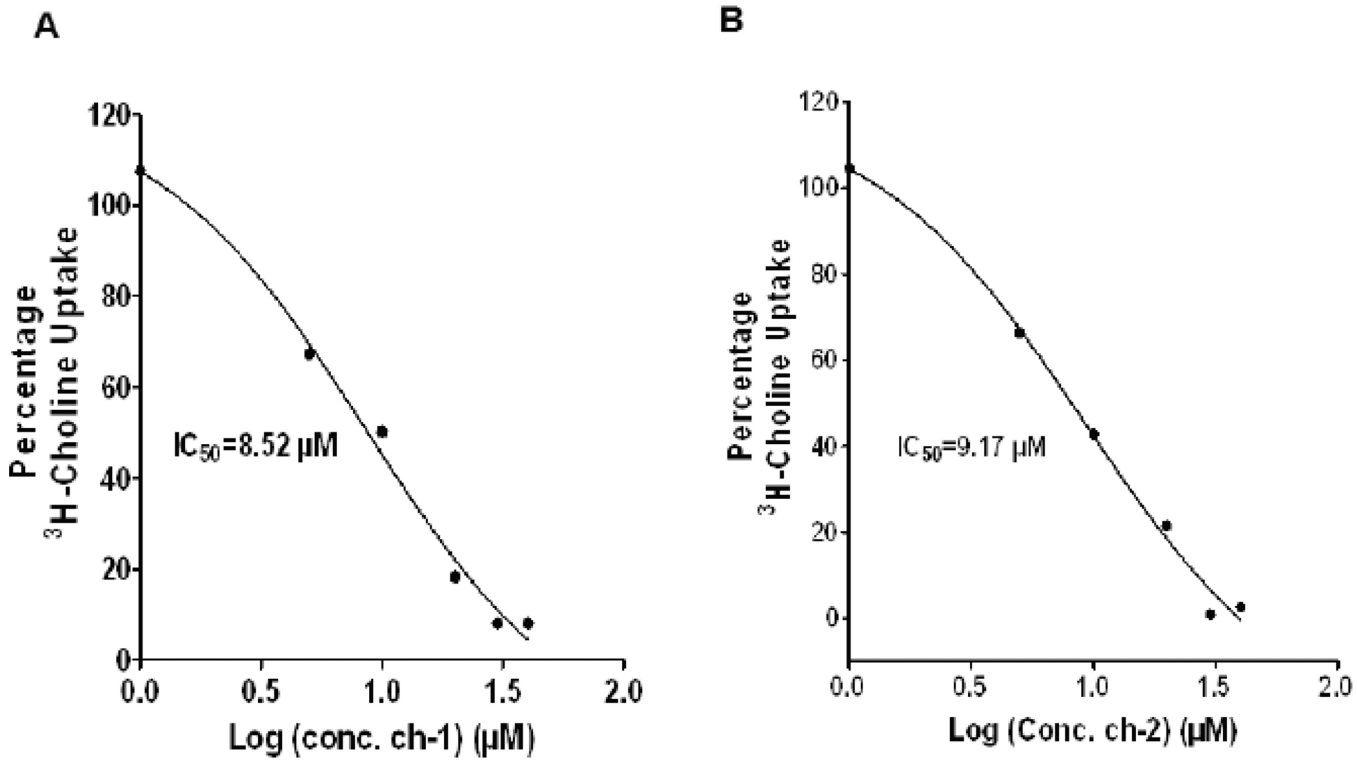

Fig. (2).

Choline transporter binding studies in MDA-MB-231Br-Luc cells. Cells were treated with ^{3}H-Choline and compounds (Ch-1 and Ch-2 separately). Ch-1 and Ch-2 compounds showed 50% inhibition of ^{3}H-Choline at 8.52±0.25 µM and 9.17±0.15 µM (**A** and **B**) respectively. Data suggests that these compounds can have the ability to bind choline transporter. Data represents Mean±SD, n=3.

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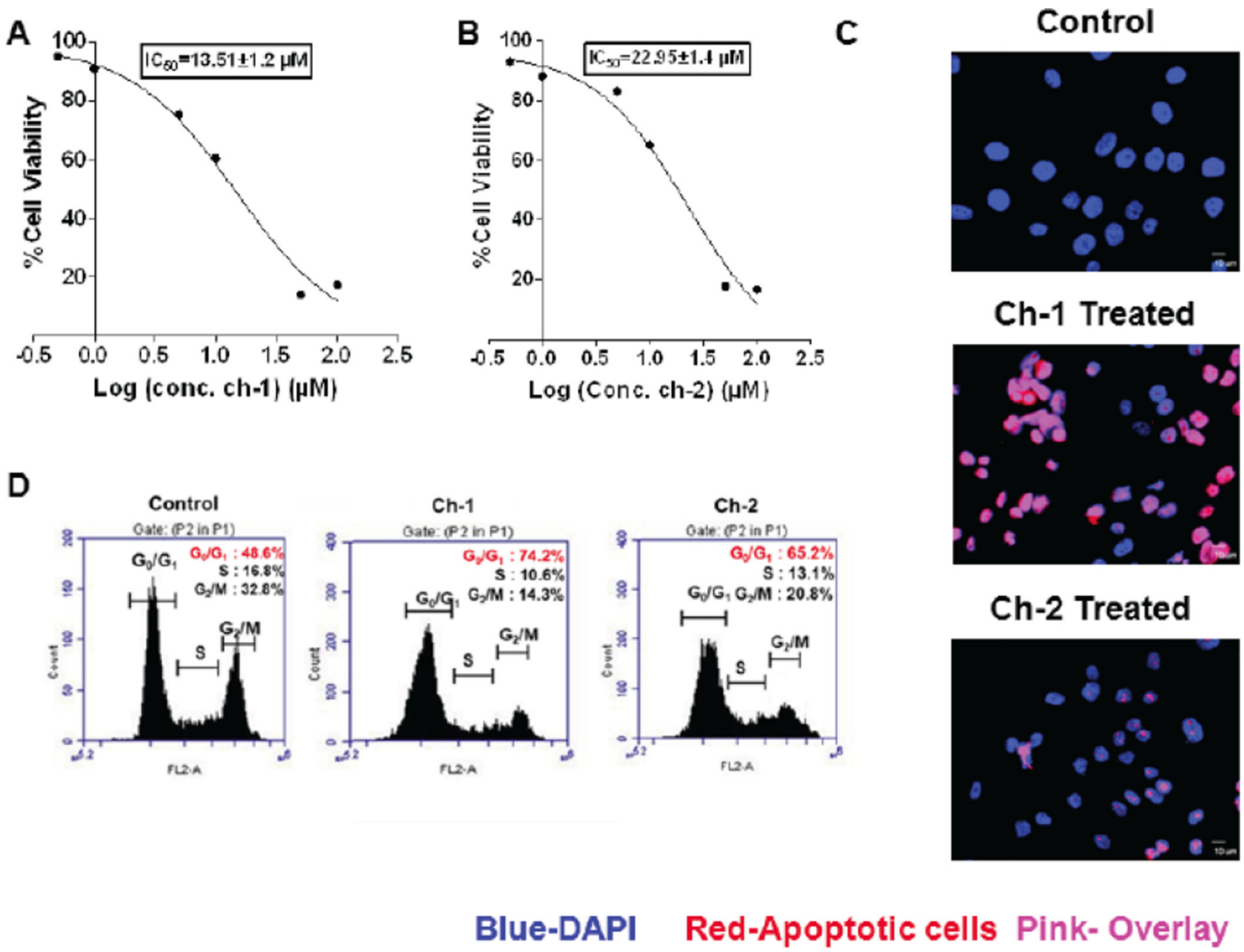

Fig. (3).

In vitro cytotoxicity, Cell cycle arrest and apoptosis in MDA-MB-231Br-Luc cells. Cytotoxicity was determined after 48h using MTT assay. IC_{50} values of Ch-1 and Ch-2 were found to 13.51±1.2µM and 22.95±1.4µM respectively (**A** and **B**). Ch-1 showed lower IC_{50} value compared to CH-2. Data suggest that Ch-1 was more toxic than Ch-2 towards MDA-MB-231Br-Luc cells. (**C**) *In vitro* apoptosis was studied in MDA-MB-231Br-Luc cells using Click-iT® TUNEL assay. Microscopic pictures suggest that Ch-1 treated showed more apoptosis compared to Ch-2. (**D**) Cell cycle arrest was studied using Propidium Iodide as marker and both the compounds showed cell cycle arrest at G_0/G_1 Phase. Data represent Mean±SD, n=3.

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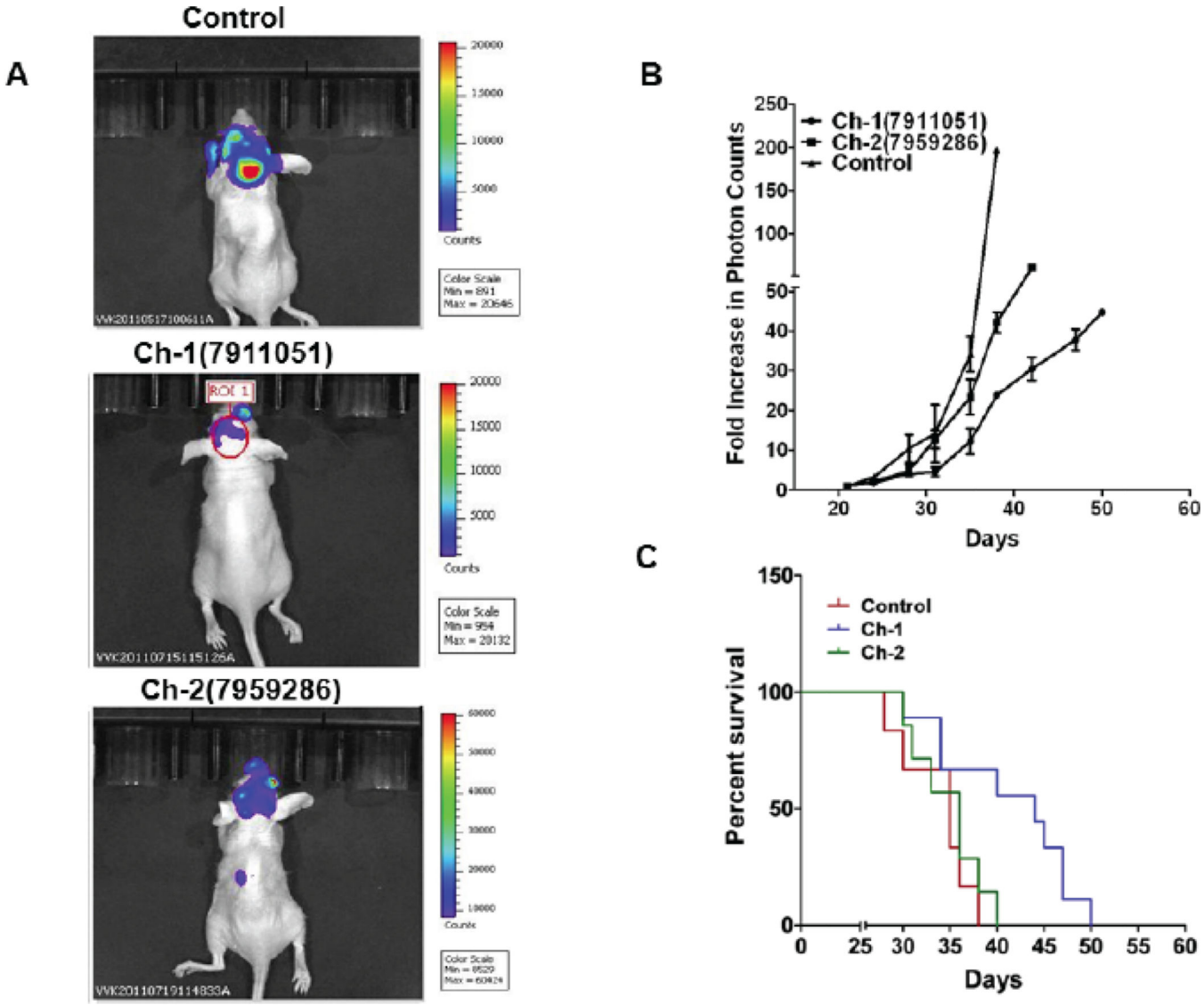

Fig. (4).

In-vivo metastatic tumors in experimental breast cancer induced brain metastasis model. (**A**) Number of metastatic tumors were reduced significantly with both compounds (Ch-1 and Ch-2) compared to control. Data represents Mean±SD, n=10 animals. (**B**) Tumors were classified into four categories based on the tumor size (Small- <0.2 mm^2, Medium- >0.2 and <0.8 mm^2 and Large -> 0.8 mm^2). Data suggest that percentage of large tumors were reduced significantly with Ch-1 compared to control and in-significant difference was observed in between other groups. Data represents Mean±SD, n=10 animals. (**C**) Representative image of terminal bioluminescent signal from an animal with experimental 231Br brain metastases with corresponding histological confirmation. (**D**) Representative images of in-vivo apoptosis in 20μm brain slice after treatment with different compounds in breast cancer induced brain metastasis model. Apoptosis was studied using Click-iT® TUNEL assay. Microscopic images suggest that both the compounds induced apoptosis but Ch-1 showed more apoptosis compared to Ch-2.

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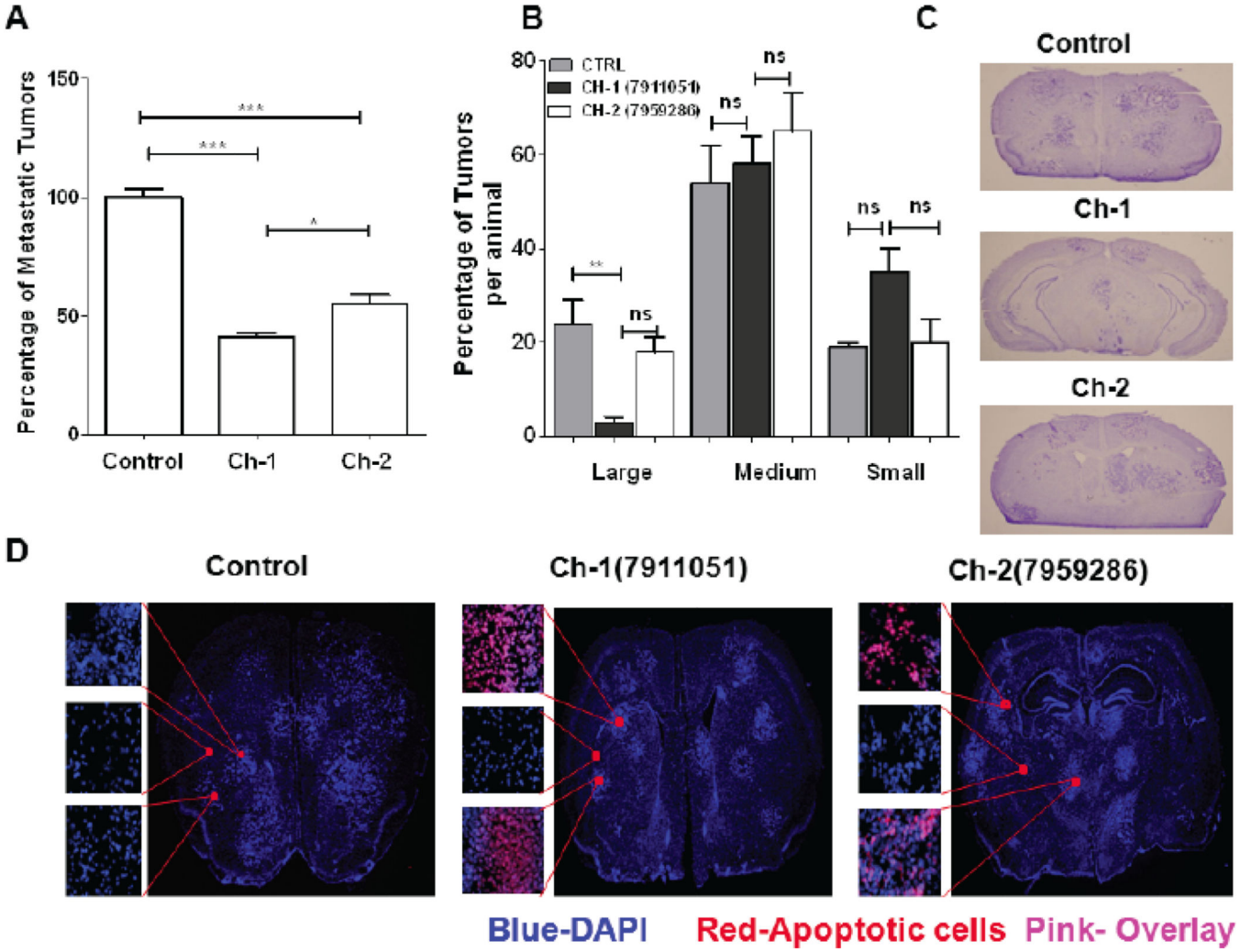

Fig. (5).

Survival study in experimental breast cancer induced brain metastasis model. (**A**) Representative bioluminescent images of animals 24 hrs after intracardiac injection of the 231Br cells. After confirmation of luminescent signal in brain, animals were randomized to either control, treatment groups (Ch-1 and Ch-2, 1mg/kg, i.p. and twice a week). (**B**) Significant decrease in tumor growth (in photons) was observed with Ch-1 compound compare with control and Ch-2 ($p<0.05$). But Ch-2 showed insignificant difference compared to control ($p>0.05$). (**C**) Median survival was prolonged as determined by the log rank test in the Ch-1 group compared to control. n=10 for all groups. (D) Brain slice staining for number of apoptotic cells in with treatment of Ch-1 and Ch-2. As can be seen in panel D, there was in increased number of apoptotic cells in the brains of mice treated with the compounds as compared to the control mouse with brain tumors.

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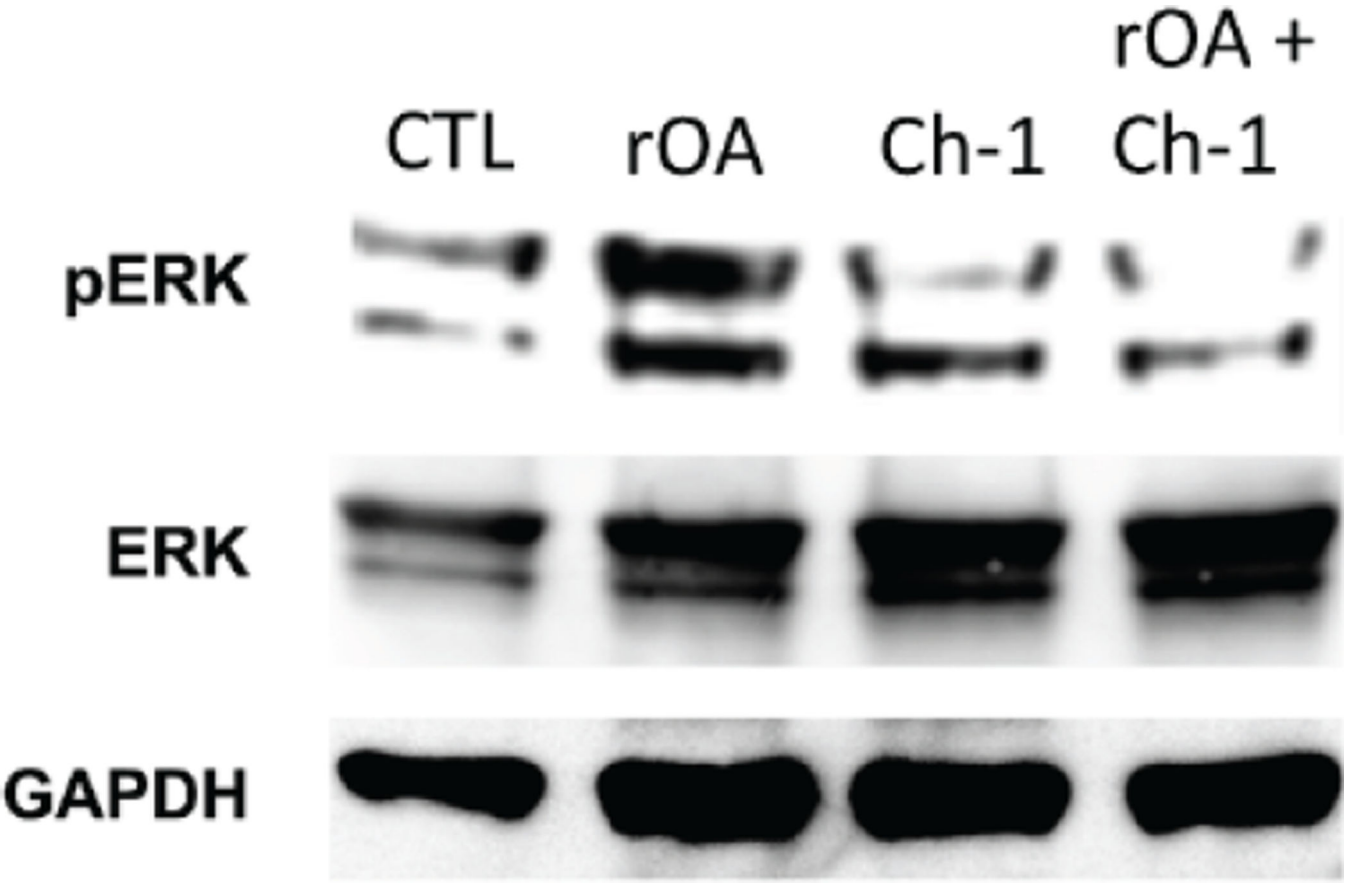

Fig. (6).

Ch-1 prevents the activation of ERK 1/2 kinase by OA (GPNMB). Cells were treated with 50 ng of OA (GPNMB) with or without Ch-1 (50 mM) for 30 min.