Molecular Cancer Therapeutics

The Rolipram–Perillyl Alcohol Conjugate (NEO214) Is A Mediator of Cell Death through the Death Receptor Pathway

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Abstract

Glioblastoma (GBM) is a highly aggressive primary brain tumor with a poor prognosis. Treatment with temozolomide, standard of care for gliomas, usually results in drug resistance and tumor recurrence. Therefore, there is a great need for drugs that target GBM. NEO214 was generated by covalently linking rolipram to perillyl alcohol (POH) via a carbamate bond to form the rolipram–perillyl alcohol conjugate. We show here that NEO214 is effective against both temozolomide-sensitive and temozolomide-resistant glioma cells. Furthermore, NEO214 is effective for different mechanisms of temozolomide resistance: overexpression of MGMT (O^6-methylguanine methyl-transferase); deficiency in specific mismatch repair proteins; and overexpression of base excision repair (BER) proteins. NEO214-induced cytotoxicity involves apoptosis triggered by endoplasmic reticulum (ER) stress, as well as activating the Death Receptor 5 (DR5)/TNF-related apoptosis-inducing ligand (TRAIL/Apo2L) pathway. *In vitro* studies show that glioma cells treated with NEO214 express DR5 and exhibit cell death in the presence of recombinant TRAIL, a growth factor constitutively produced by astrocytes. Our *in vitro* 3D coculture data show that induction of DR5 in glioma cells with NEO214 and TRAIL cause tumor cell death very effectively and specifically for glioma cells. *In vivo* studies show that NEO214 has antitumor efficacy in orthotropic syngeneic rodent tumor models. Furthermore, NEO214 has therapeutic potential especially for brain tumors because this drug can cross the blood–brain barrier (BBB), and is effective in the TRAIL-rich astrocyte microenvironment. NEO214 is a strong candidate for use in the treatment of GBMs.

Introduction

Glioblastoma (GBM) is a highly vascular and invasive primary brain tumor (1). Treatment with the standard-of-care chemotherapy, temozolomide, usually results in drug resistance and ultimately tumor recurrence (2, 3). Therefore, there is an ever increasing need for the development of nontoxic, effective drugs that specifically target chemoresistant gliomas. NEO214, the conjugate of rolipram and perillyl alcohol (POH), was synthesized on the basis of the properties of these components. Rolipram is a selective inhibitor of the enzyme phosphodiesterase 4 (PED4; ref. 4), which increases cyclic AMP (cAMP) levels by inhibiting the breakdown of cAMP to adenosine by phosphodiesterases. Increased cAMP inhibits proliferation and can also stimulate apoptosis (5). It also has been reported that rolipram crosses the blood–brain barrier (BBB) and has antitumor activity (5, 6). The mechanisms associated with antitumor activity of phosphodiesterase 4 inhibitors have been shown to decrease angiogenesis and inhibit inflammatory cell migration (6, 7). Perillyl alcohol (POH) is a naturally occurring monoterpene and constituent of caraway, lavender, and lilac oil, as well as other plants. It was initially demonstrated to be a cell-cycle inhibitor (8, 9). As shown in several clinical trials, when this drug was administered orally at high doses, it induced significant gastrointestinal toxicity, without significant tumor inhibition. POH has also been administered intranasally for intracranial neoplasms with some success (10, 11). NEO100, GMP quality POH, has been approved for fast track and orphan drug status by the FDA, and is currently in a phase I/IIa trial for patients with first recurrence GBM after the Stupp protocol (2). Studies from our laboratory have also shown that POH was able to cross the BBB (12–14). On the basis of these anticancer properties of rolipram and POH, the novel conjugate NEO214 was synthesized and tested for efficacy.

Understanding the molecular pathways that are implicated in cell death of cancer cells is essential for developing novel therapeutic approaches. Our studies show that the mechanism of NEO214-induced cytotoxicity is triggered by way of at least two unique pathways: endoplasmic reticulum (ER) stress and Death Receptor 5 (DR5) activation. The ER is a specialized organelle required for protein folding and Ca^{2+} storage. Glioma cells react to ER stress by initiating the unfolded protein response (UPR) to

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restore homeostasis; however, if stress persists, the UPR induces apoptotic cell death (15). Among the key proteins of the apoptotic pathway is CHOP (CCAAT/enhancer binding protein homologous transcription factor also called GADD153), a transcription factor that alters the transcriptional profile of cells and triggers a proapoptotic program (16). Prolonged expression of CHOP is a key trigger for apoptosis, and therefore this protein is commonly used as a marker to indicate that proapoptotic events of the UPR have been initiated (17). ER stressors induced DR5 transcription via the UPR mediator CHOP. Death receptors (DR) 4 and 5 are members of the TNF receptor superfamily (18, 19) and are expressed in a broad range of cancer cells. The binding of the death ligand TNF-related apoptosis-inducing ligand (TRAIL/APO-2L) to cell-surface death receptors results in trimer formation of DR, followed by recruitment of the adaptor protein FAS-associated protein with death domain (FADD) and the formation of the death-inducing signal complex (DISC). Subsequently, the initiator caspase 8 or 10 is recruited and triggers the apoptotic signal (20).

Here we demonstrate that NEO214 triggers cell death through ER stress by causing increased calcium release into the cytoplasm, thus upregulating the DR5, a trigger for the extrinsic apoptosis pathway when it is activated by its ligand, TRAIL. NEO214 induces DR5 on a select population of cells that can be induced to express DR5, particularly tumor cells, without affecting the nonmalignant cells tested. In the brain, TRAIL is constitutively secreted by astrocytes (21); these nonmalignant astrocytes do not express DR5 upon treatment with NEO214. Thus, this process is selective for tumor cells, as with normal cells there is no cell death. However, the brain microenvironment provides the appropriate environment for NEO214 activity. The presence of NEO214 in the brain microenvironment results in enhanced cell death of tumor cells. These data show that NEO214 is an effective antitumor agent that is specifically active in the brain microenvironment.

Materials and Methods

Cells and reagents

All human tissue specimens were obtained in accordance with the Institutional Review Board guidelines established at Keck School of Medicine, University of Southern California (Los Angeles, CA). The human glioma cell line, U251, and temozolomide-resistant cell lines, U251TR, LN229TR, and T98G, were cultured in DMEM supplemented with 10% FCS, 100 U/mL penicillin and 0.1 mg/mL streptomycin, in a humidified incubator at 37°C and 5% CO_2. Human glioma cell lines were purchased from ATCC; the cell lines have been authenticated. Temozolomide-resistant cells were developed in this laboratory, as described previously (22). Briefly, cell lines were incubated with increasing doses of temozolomide ranging from 10 to 100 μmol/L for approximately 6 months; the drug was administered daily. Once the cells became temozolomide-resistant, these cell lines were treated with 100 μmol/L temozolomide every other week. This method of developing temozolomide-resistant cell lines was designed to parallel the development of temozolomide resistance in the human population, where GBM populations are initially sensitive and then develop resistance. This phenotype has been stable for over 2 years. Human patient-derived glioma cells were isolated from freshly resected GBM tissues obtained according to the University of Southern California (USC, Los Angeles, CA) Institutional Review Board guidelines. Information regarding the detailed protocol and culture medium has been previously published (23). Patient-derived glioma cells were cultured in cancer stem cell culture (CSC) medium containing DMEM-F12 medium (Life Technologies) with 1% penicillin–streptomycin, 1% B-27 (Life Technologies), and 20 ng/mL EGF and bFGF (PeproTech). Human astrocytes were purchased from ATCC and cultured in astrocyte medium (ScienCell). Human brain endothelial cells (BEC) were isolated and characterized as described previously (24). These cells were cultured in RPMI containing 10% FCS supplemented with Endogro (Millipore), and used only up to passage 6. Endogro was not used during the *in vitro* experimental procedure.

NEO214 was provided by NeOnc Technologies, Inc. This reagent was prepared as 100 mmol/L stock solution in DMSO, and stored at −20 °C. NEO100 (NeOnc Technologies, Inc.) was purified and provided as 6,500 mmol/L stock solution and stored at 4°C (12). Rolipram (Sigma Aldrich) was prepared as 100 mmol/L stock solution in DMSO and stored at −20 °C. Recombinant human TRAIL/Apo2 ligand (PeproTech), was prepared as 1 mg/mL stock solution in water and stored at 20°C with 10 or 20 μL aliquots to avoid the freeze and thaw cycle. The above drugs were added to culture medium achieving the final concentration of DMSO to be less than 0.01%–0.4%. There were no differences in media control cultures in the presence or absence of DMSO.

Alamar blue assay

Glioma cells (1,000 cells/well), astrocytes, or BEC (10,000 cells/well) were seeded in 96-well assay black plates with flat bottom (Greiner). After 24 hours, different concentrations of drug were added to the cells, and incubated for 48 to 72 hours. The Alamar blue assay was performed according to the manufacturer's protocol (Life Technologies). Fluorescence was measured using excitation wavelength of 540–570 nm (peak excitation is 570 nm), emission at 580–610 nm (peak emission is 585 nm). The average fluorescence values of cell culture medium alone (background) were subtracted from the fluorescence values of experimental wells. Percent survival was calculated relative to untreated control cells. All experiments were performed in triplicate.

Colony-forming assay

Glioma cells were seeded in 6-well plates at 200 cells/well and allowed to adhere overnight. Subsequently, cells were treated with drugs for 48 hours; the medium was then removed and fresh medium (with no drug) was added. Cells were incubated for an additional 7–10 days. At the termination of the assay, colonies were visualized by staining with 1 % methylene blue in methanol for 4 hours. Dyes were washed out with water and then air dried. The stained colonies were counted. Percent colonies were calculated relative to untreated control cells. All experiments were performed in triplicate.

NEO214 drug stability test:

NEO214 (100 mmol/L stock prepared in DMSO) was diluted into the medium to 50 μmol/L and incubated at 37 °C water bath for 0, 0.5, 1, 2, 4, 24, 48, 72, and 96 hours and then used to test for cytotoxic activity with CFA as described above.

Western blot analysis

Total cell lysates were prepared by disrupting cells with RIPA buffer; protein concentrations were determined using the BCA Protein Assay Reagent (Pierce). Fifty μg of total cell lysate was

added to each lane; 10%, 12.5%, or 15% of SDS-PAGE gels were used according to the size of protein of interest. Trans-blot (Bio-Rad) was used for the semi-dry transfer. Antibodies to GRP78 and actin were obtained from Santa Cruz Biotechnology, Inc. and used according to the manufacturer's recommendations. Antibodies to CHOP, cleaved caspase-7, and PARP were purchased from Cell Signaling Technology. Antibodies to DR5 were purchased from R&D Systems. Anti-DR4 and anti-TRAIL were purchased from Abcam. Horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology) were used for detection.

Transfection with siRNA

Cells were placed in 6-well plates in serum-free media and transfected using the Lipofectamine 2000 Kit (Life Technologies) according to the manufacturer's instructions. Five nanomoles of siRNA were used for the knockdown. The different siRNAs (si-GFP and si-CHOP) were designed and synthesized by Ambion (Life Technologies). Transfected cells were seeded for the evaluation of long-term survival after drug treatment or harvested for Western blot analysis (to confirm knockdown of target gene expression).

Knockdown with DR5 shRNA lentiviral particles

Lentiviral particles were purchased from Santa Cruz Biotechnology. Cells were placed in 6-well plates at 30%–40% confluency, the day before transduction. Medium, polybrene (linker molecule, 8 μg/mL), and viral particle (final 1× concentration) were mixed and added to each well; cells were incubated for 48 hours. Fresh media were added and wells were incubated for another 24 hours. Transduced clones were selected by treating with puromycin for 24–48 hours. Selected transduced cells were seeded for the evaluation of survival after drug treatments or harvested for Western blot analysis to confirm the knockdown of target gene.

Measurement of intracellular calcium

Free intracellular calcium was determined by using Fluo-3AM (Invitrogen). U251 cells were seeded on coverslips the day before the experiment and treated with NEO214 for 20 hours. Cells were then stained with Fluo-3 dye containing medium for 1 hour at 37°C. The dye was then washed out with dye-free media and fixed with 3.7% formaldehyde for 30 minutes. Coverslips were mounted on glass slides; and intracellular calcium levels were determined by monitoring fluorescence inside the cells by fluorescence microscopy.

Isolation of microsomes from skeletal muscle

Skeletal tissue muscles from mice were isolated and washed with ice-cold PBS. Subsequently 1× isotonic extraction buffer (with phosphatase and protease cocktail) per gram of tissue was added and tissue cut with the aid of scalpel in plastic plate. Tissues were tansfered to a 50 mL tube, and homogenized by moving pestle up and down at least 7 times (total 10 mL). The homogenate was centrifuged at 1,000 × *g* for 10 minutes at 4°C. Supernatant was carefully transferred to different tubes and the thin floating lipid layer was removed. The supernatant was centrifuged at 12,000 × *g* for 15 minutes at 4°C, the supernatant was carefully transferred, and the thin floating lipid layer was removed (this is PMF: postmitochondrial fraction). The PMF was centrifuged for 60 minutes at 100,000 × *g* in an ultracentrifuge at 4°C. The pink pellet is the microsomal fraction. The pellet was resuspended in 1× isotonic buffer and the protein concentration was measured. Microsomes were stored at −80 °C or used immediately. All the reagents were prepared according to a previously published article (25).

Measurement of sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) activity

The reaction mixture [total 500 μL: 5× reaction buffer: 100 μL, 2 mmol/L $CaCl_2$: 50 μL, water: 300 μL, microsome: 20 μg (2–5 μL)] was prewarmed in a 37°C waterbath for 5 minutes. The reaction was initiated by adding 10 mmol/L ATP (50 μL). The reactions were stopped by sampling (50 μL) at different time points and mixing with stop solution (200 μL). The ODs were read at 630 nm. All the reagents were prepared according to a previously published article (25).

IHC

Frozen tissues were sectioned, then fixed in acetone, washed with PBS, and blocked with Sea Block (Thermo Scientific) for 20 minutes. Subsequently, IHC was performed as described previously (26). For antigen retrieval, tissue sections were fixed in 10% neutral buffered formalin for 10 minutes and stained as published previously (27). Antibodies recognizing O^6-methylguanine methyltransferase (MGMT) were purchased from Abcam.

3D coculture assay

Normal human astrocytes were seeded on coverslips in 24 well plates at 1×10^5 cells/well and incubated for 24 hours. Subsequently, GFP-labeled U251 tumor cells (5×10^3 cells/10μL) were applied on top of the astrocytes and cultured for an additional 24 hours. At this point, 50 μmol/L of NEO214 or vehicle was added to the cocultures and incubated for another 24 hours. The coverslips with cocultured cells were rinsed with PBS and fixed in 4% formalin for 20 minutes and stored at 4°C.

Immunofluorescence staining

Cells grown on coverslips were incubated with 0.3% Triton X-100 (in PBS) for 30 minutes at 37°C. Subsequently, the slides were rinsed in PBS for an additional 5 minutes and blocked with SEA block blocking buffer (1:1 in PBS) for 1 hour at room temperature. Primary antibodies for GFAP (Santa Cruz Biotechnology) or TRAIL (1:100) were added to the coverslips for overnight incubation at 4°C. The next day, slides were washed with PBS and incubated with the appropriate AlexaFluor secondary antibody (Life Technologies) at the recommended concentration for 1 hour. The images were obtained using confocal microscopy.

TRAIL ELISA assay

Equal numbers of cells were cultured in media for 48 hours; and supernatants were collected and analyzed for TRAIL using the Human TRAIL Immunoassay kit (R&D Systems), according to the manufacturers' protocols. The samples were plated in duplicates. The data are presented as quantity of TRAIL per mL. All experiments were performed in triplicate.

***In vivo* intracranial glioma rodent model**

All animal protocols were approved by the Institutional Animal Care and Use Committee of USC; all rules and regulations were followed during experimentation. Intracranial implantation of tumor cells was performed as described previously (22). Athymic nude mice were anesthetized and implanted with 2×10^5 luciferase-positive human glioma cells (U251) into the frontal lobe. Mice were imaged 7–10 days postimplantation. Once tumors

appeared in all the mice, as determined by imaging, animals were randomly placed into different groups ($n = 4$) and treatment began. NEO214 at 50 mg/kg was diluted in vehicle (50% glycerol/50% ethanol) and administered subcutaneously in a volume of 100 μL. Tumor growth was monitored by imaging and the survival was documented.

To test NEO214 using the syngeneic intracranial tumor model, 6- to 8-week-old female C57bl/6 mice (Harlan, Inc.) were anesthetized, and implanted with 1×10^5 mouse glioma cells (GL261) into the frontal lobe. After 7 days postimplantation, animals were randomly placed into different groups ($n = 4$) and treatment began. NEO214 at 100 mg/kg was diluted in vehicle (50% glycerol/50% ethanol) and administered subcutaneously or by gavage in a volume of 100 μL. Mouse survival was recorded. For pathology analysis and drug toxicity analysis, C57bl/6 mice were treated with NEO214 at 100 mg/kg diluted in vehicle (50% glycerol/50% ethanol) and administered subcutaneously for 20 days, several organs (i.e., spleen, liver, kidney, intestines, heart, and lung), bone marrow, and blood were harvested. Organ samples were fixed in 10% formalin, paraffin embedded, sectioned, and stained with hematoxylin and eosin. Bone marrow specimens were removed from the bone, smeared on to the slide, and stained with Hemacolor stain (EMD Millipore). Blood samples were collected and analyzed for white blood cells, red blood cells, and platelets (Antec). To detect CHOP and DR5 expression in the syngeneic mouse brain tumor model, 1×10^5 mouse glioma cells (GL261) were implanted into the frontal cortex. Ten days postimplantation, mice were treated with NEO214 (100 mg/kg) subcutaneously once, and mouse brains were harvested after 24, 48, and 72 hours, and stained for Ki67, CHOP, and DR5.

Statistical analysis

Statistical significance was evaluated using the Student two-tailed *t* test for all *in vitro* experiments. The log-rank test was used to evaluate significance for the survival curve; *P* values <0.05 were considered significant.

Results

NEO214 is cytotoxic for temozolomide-resistant glioma cells and is more potent than either temozolomide or POH alone or the mixture of these two components

NEO214 was synthesized by covalently linking perillyl alcohol (POH) to rolipram using a carbamate bond (Fig. 1A). NEO214 was tested for cytotoxic activity using temozolomide-sensitive (U251; Fig 1B), temozolomide-resistant glioma cell lines: U251TR (Fig 1C), LN229TR (Fig 1D), and T98G (Fig. 1E) cells, as compared with temozolomide alone. The data show that the IC_{50} for NEO214 and temozolomide is independent of mode of temozolomide resistance. Temozolomide-sensitive cells (U251) were similar to temozolomide-resistant cells (30 μmol/L). However temozolomide-resistant glioma cells were significantly more sensitive to NEO214 as compared with temozolomide alone. The colony-forming assay (CFA) was used as the assay system for Fig. 1B–E. Furthermore, the results show that NEO214 is cytotoxic for MGMT-positive temozolomide-resistant GBM cells, such as T98G cells. The IC_{50} for T98G cells treated with NEO214 is 40 μmol/L, while these cells treated with temozolomide is >100 μmol/L. Thus MGMT-positive patients may effectively be treated with this drug (28). We also showed that NEO214 was cytotoxic for patient-derived tumor cells (Fig. 1F), including USC02, which are MGMT positive, while other cell populations (USC04 and USC10) are MGMT negative. These data show that NEO214 cytotoxic activity is independent of MGMT expression status. In Fig. 1G, we tested GL261, the mouse glioma cell lines used here for the *in vivo* syngeneic intracranial tumor model. The results showed that NEO214 is cytotoxic to GL261 as IC_{50} of 50 μmol/L, which is in the similar range to human glioma cell lines. To test whether NEO214 activity is the result of the conjugate formation or mixture of the activity of two agents (rolipram and POH), cells were treated with equimolar concentrations of each drug alone, the mixture of rolipram plus POH, or NEO214 (Fig. 2A–D). On the basis of IC_{50} using the CFA, the results show that NEO214 were significantly more effective in inducing cell death in temozolomide-sensitive (U251) and temozolomide-resistant GBM cells (U251TR, LN229TR, and T98G), as compared with the components alone or the mixture of the two components.

To determine the stability of the drug *in vitro*, NEO214 (50 μmol/L) was incubated in the medium at 37°C for different times in the absence of cells. Subsequently, the drug was used to treat glioma cells (U251) *in vitro* in the CFA assay. Fig. 2E shows high cell death up to 48 hours. However, when NEO214 was incubated with cells after 48 hours of drug exposure in 37°C, cell survival was low. These results show that NEO214 has cytotoxic activity, is functionally stable, and can be maintained up to and including 48 hours. After 72-hour incubation in media, the drug was no longer cytotoxic.

NEO214 triggers apoptosis by activating the ER stress pathway

To identify the mechanisms responsible for NEO214-induced cell death, glioma cells were treated with increasing concentrations of NEO214 for 20 hours (Fig. 3A). The data show that NEO214 increased the expression of CHOP, a transcription factor responsible for cell death resulting from ER stress (29), and GRP78, a measure of the unfolded protein response (UPR), characteristic of ER stress (30, 31). Cleaved caspase-7, a downstream cell death effector caspase, was also increased. We also tested the induction of ER stress markers in GL261 cells treated with NEO214; GRP78, and CHOP were upregulated in this mouse glioma cell line (Fig. 3B). To determine whether calcium plays a role in NEO214 triggering ER stress, glioma cells were treated with NEO214 (50 μmol/L) for 20 hours, then evaluated for calcium release using the calcium detection dye, Fluo-3AM dye (Fig. 3C). The results demonstrated that NEO214 caused a release of calcium into cytoplasm; no significant fluorescence was detected in untreated cells. The ER is a the major cellular Ca^{2+} store where ER luminal Ca^{2+} concentration is balanced between sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) and passive Ca^{2+} leak. To measure the SERCA activity, we isolated liver microsomes from mouse liver and measured SERCA activity in the absence or presence of NEO214 (Fig. 3D). The results showed that NEO214 did not inhibit SERCA activity, as compared with the inhibitory effects of thapsigargin, which is known to be a SERCA inhibitor (positive control; ref. 32). We then examined the effects of NEO214 on a passive Ca^{2+} leak. Previous studies have shown that the translocon (TLC), a protein complex involved in protein translocation during translation (33), is an ER Ca^{2+} leak channel (34, 35). In the presence of anisomycin, the translocon inhibitor (36), no free Ca^{2+} was detected in the cytoplasm. As shown in Fig. 3E, NEO214 (N) alone increased intracellular Ca^{2+} concentration; while anisomycin together with NEO214 (A+N) greatly reduced intracellular Ca^{2+}. To determine whether this

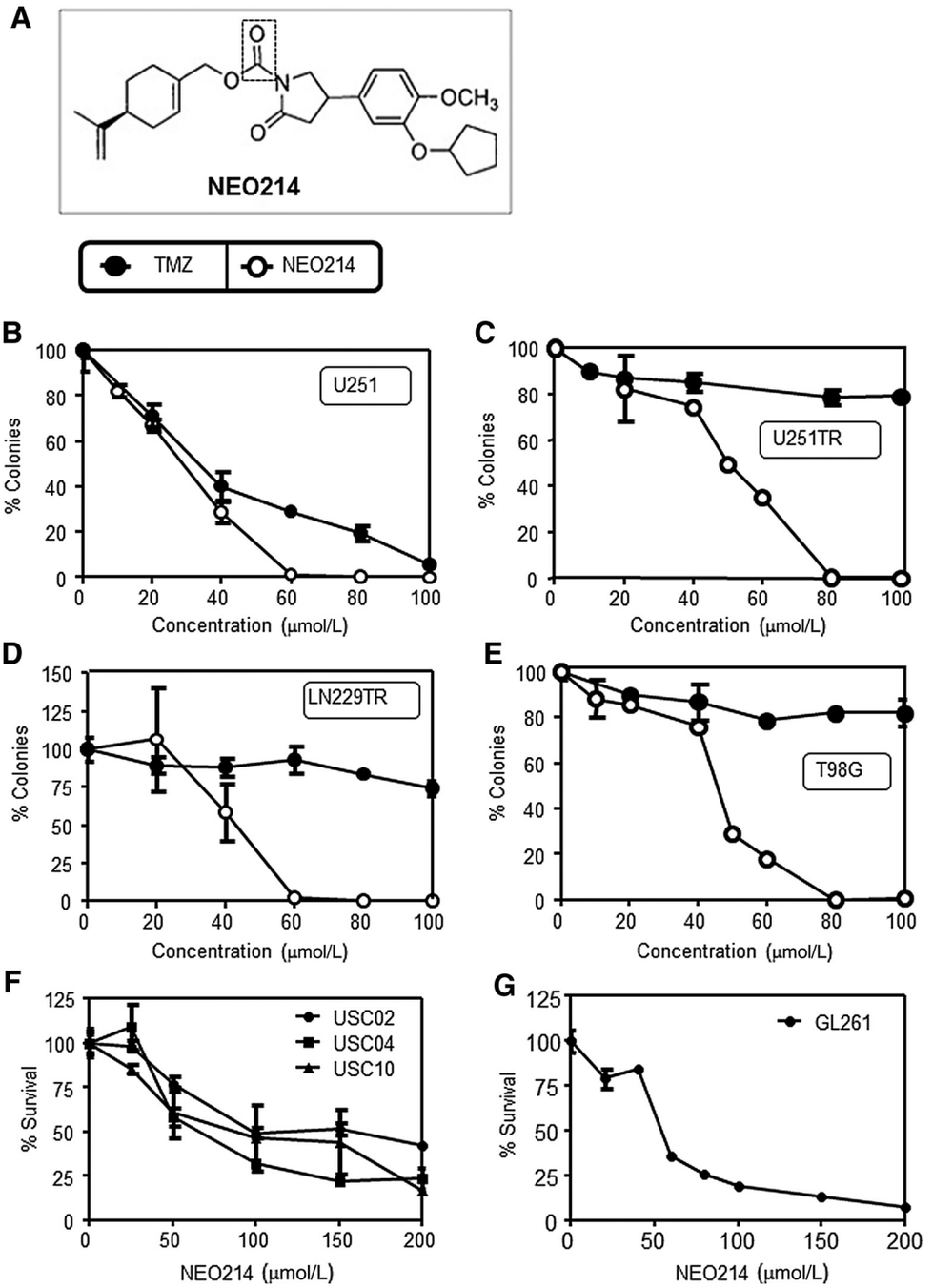

Figure 1.

Structure and cytotoxic effects of NEO214 on temozolomide (TMZ)-sensitive and temozolomide-resistant glioma cell lines. **A,** The chemical structure of NEO214. Rolipram is covalently linked to POH via carbonyl bridge (dotted box). **B,** Temozolomide-sensitive cell line (U251) and temozolomide-resistant cell lines: U251TR (**C**), LN229TR (**D**), and T98G (**E**) were exposed to increasing concentrations of temozolomide (black circle) and NEO214 (white circle) for 48 hours, then cultured for another 7–10 days in fresh medium in the colony formation assay (CFA). NEO214 was cytotoxic to a range of glioma cells with an IC_{50} between 40 and 60 μmol/L. **F,** Patient-derived primary tumor cells treated with NEO214. **G,** GL261 was treated with NEO214. Experiments were performed in triplicate.

release of Ca^{2+} was associated with NEO214-induced CHOP, Western blots for CHOP were performed (Fig. 3F). With NEO214 treatment (N), tumor cells induced CHOP and GRP78; however, with cotreatment of anisomycin (A+N), CHOP and GRP78 expression decreased. These experiments were confirmed using medium with or without calcium. Our results show that NEO214 triggers ER stress in tumor cells mainly through Ca^{2+} leak mediated by the translocon (TLC). Blocking intracellular Ca^{2+} leak

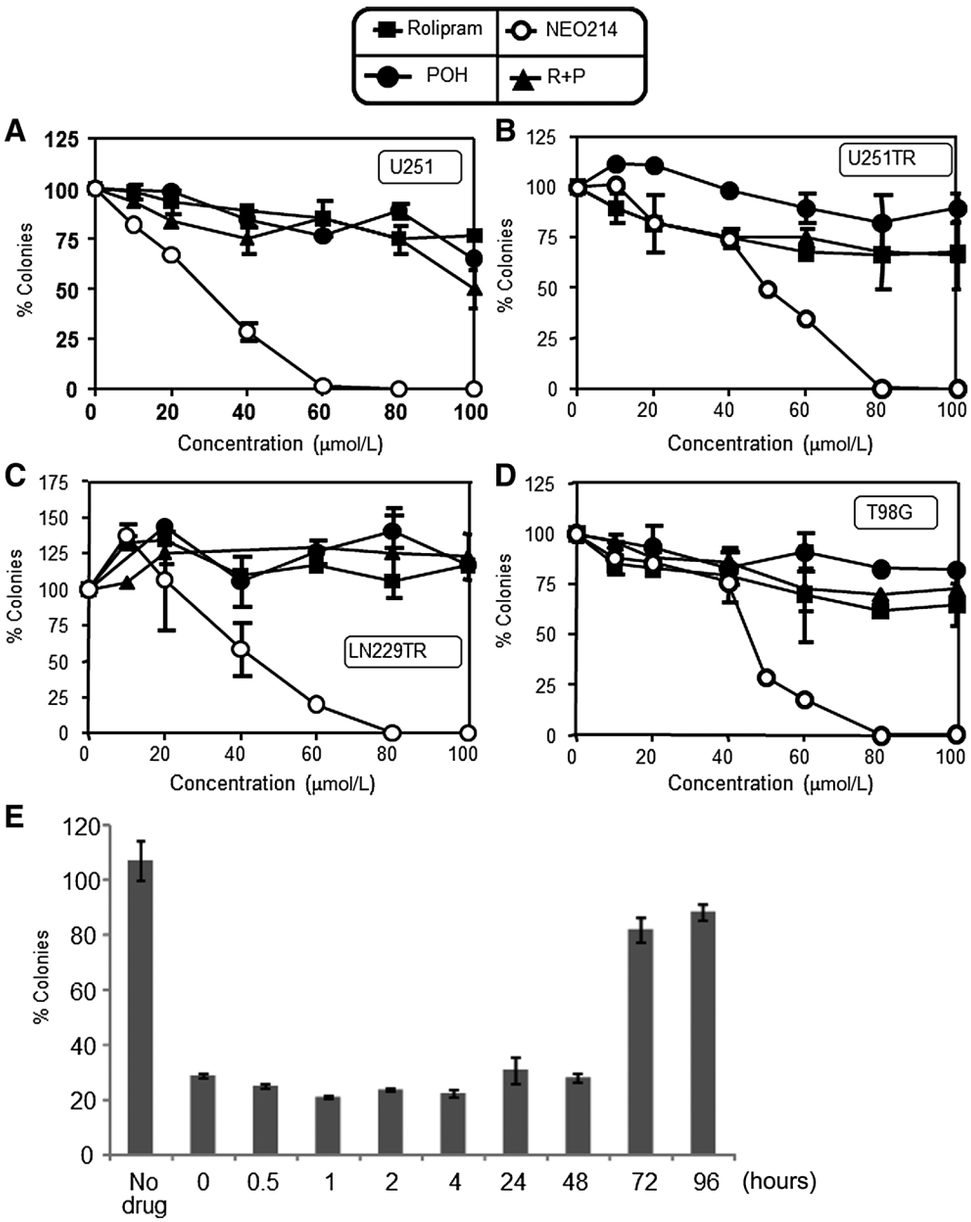

Figure 2.

NEO214 conjugate is more cytotoxic than the individual component or mixture of the two components. Temozolomide (TMZ)-sensitive U251 (**A**) and temozolomide-resistant cell lines: U251TR (**B**), LN229TR (**C**), and T98G (**D**) were tested in CFA; cells were exposed to increasing doses of NEO214 (open circle), rolipram (closed square), POH (closed circle), or equimolar concentrations of rolipram plus POH (closed triangle). After 48 hours of treatment, cells were cultured for additional 7–10 days in fresh medium. The percent colonies was derived from the ratio of vehicle control compared with treated cells. The data were calculated on the basis of two independent experiments ($n = 2$). **E,** The stability of NEO214 after incubating the drug in the 37°C medium. Cytotoxic activity of NEO214 was measured with CFA assay.

through TLC by anisomycin blocks the ER stress induction by NEO214. To further confirm that cell death with NEO214 was due, at least in part, to ER stress, cells were transfected with si-CHOP (Fig. 3G). Western blots show that si-CHOP was effective in reducing CHOP. To determine whether the CHOP expression induced by NEO214 was indeed responsible for cell death, si-CHOP glioma cells were treated with NEO214 and tested for cytotoxicity. The results showed that reducing CHOP significantly reduced the cytotoxic effects of NEO214 (Fig. 3H). The knockdown of the proapoptotic ER stress component CHOP resulted in an increase in chemoresistance as compared with cells transfected with the control si-GFP. These results demonstrated that CHOP and the ER stress pathway are critical for NEO214-induced cell death. We also found that the knockdown of CHOP downregulates DR5 expression (Fig. 3G), showing that CHOP and DR5 expressions are interdependent.

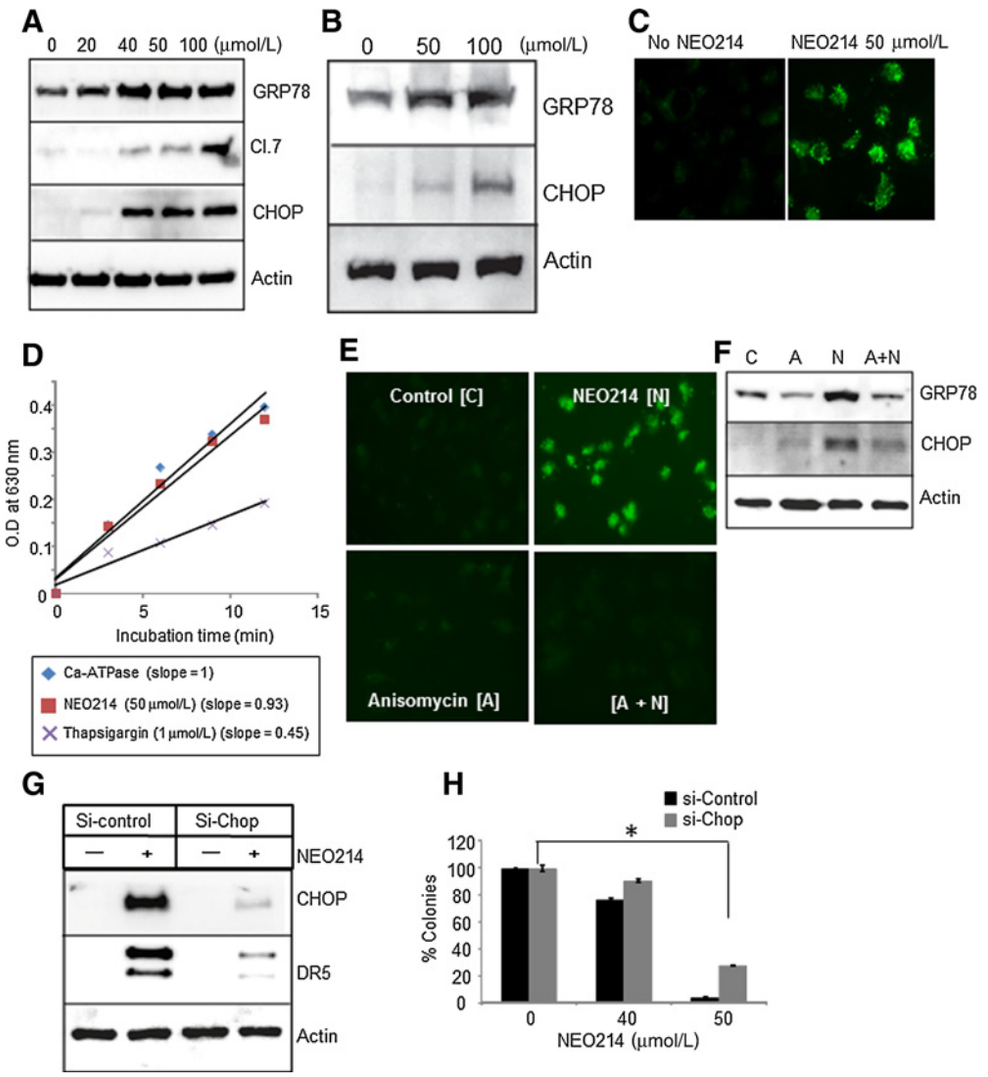

Figure 3.

NEO214 induces ER stress–mediated apoptosis. **A,** U251 were treated with increasing doses of NEO214 for 20 hours and Western blots were performed evaluating GRP78, CHOP, and cleaved caspase 7. **B,** GL261 cells were treated with NEO214 for 20 hours and Western blots were performed evaluating GRP78 and CHOP. **C,** Intracellular Ca^{2+} release is evaluated using Fluo-3AM. Cells were treated without or with NEO214 50 μmol/L for 20 hours. Cytoplasmic calcium was stained using Fluo-3AM and analyzed under fluorescent microscope. **D,** Liver microsomes were isolated from mouse liver and SERCA enzyme activity was measured. The basal Ca^{2+}-ATPase activity was measured, and its activity was compared with NEO214 (50 μmol/L) and Thapsigargin (1 μmol/L) treatment. NEO214 showed no SERCA inhibition activity. **E,** Intracellular Ca^{2+} staining with anisomycin treatment was quantified. Cells were treated with no drug control [C], anisomycin [A] (0.2 μmol/L), NEO214 [N] (50 μmol/L), or anisomycin+NEO214 [A+N] for 20 hours; intracellular calcium was stained with Fluo-3AM. **F,** Western blot analysis was performed using antibodies for the ER stress markers; GRP78 and CHOP with no drug control [C], anisomycin [A], NEO214 [N], and anisomycin +NEO214 [A+N] treatments. **G,** U251 cells were transfected with si-CHOP; si-GFP was used as the negative control. Efficiency of knockdown was confirmed by Western blot analysis. After transfection, cells were treated with 50 μmol/L NEO214 and Western blots were performed for expression of CHOP and DR5. **H,** Transfected cells were treated with NEO214 40 μmol/L and 50 μmol/L for 48 hours. Cell survival was determined by the colony formation assay (CFA). Data shown are percent colony formation compared with control ($n > 3$; **, $P < 0.005$).

NEO214 triggers DR5 and TRAIL/Apo2L-mediated cytotoxicity

Studies have shown that CHOP is responsible for the expression of the death receptors (DR4/5), which results in the triggering of apoptosis through the extrinsic apoptotic pathway (37, 38). Western blot analysis of glioma cells treated with NEO214 indicated that NEO214 upregulates DR5 expression on tumor cells. Furthermore, blocking CHOP expression blocks DR5 expression (Fig. 3G). To determine whether NEO214 directly regulates the expression of DR5, a time course study of protein expressions was performed using NEO214 (50 μmol/L) treated glioma cells (Fig. 4A). The results showed that starting from 6 hours, both activating transcription factor 3 (ATF3) and CHOP were strongly induced; and only after 12 hours was DR5 expressed. ATF3 is a known marker of ER stress activation (39). Glioma cells treated with varying doses of NEO214 exhibited an upregulation of DR5 (Fig. 4B) starting from 40 μmol/L of NEO214 in 24 hours.

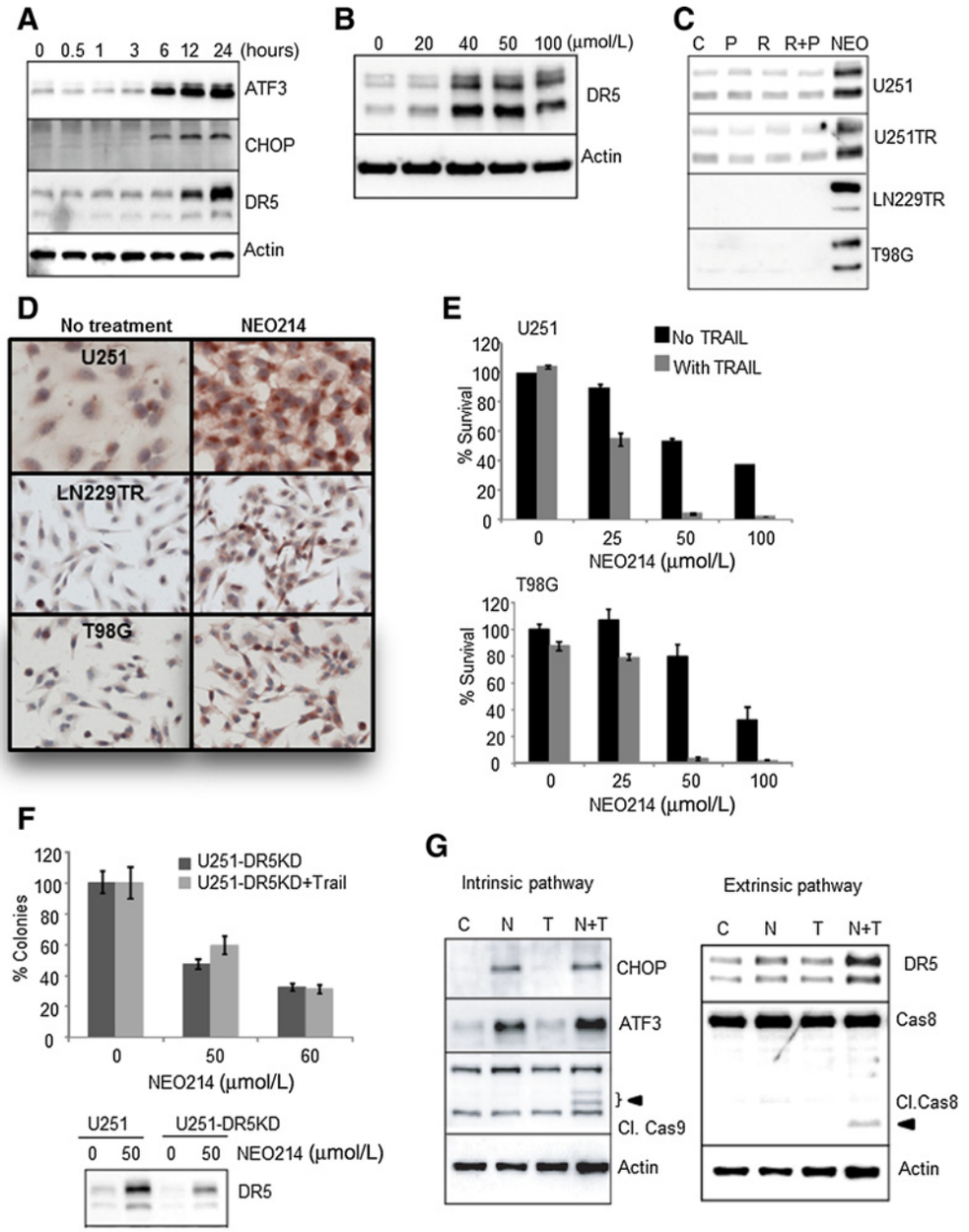

Figure 4.

NEO214 induces TRAIL/DR5-dependent apoptosis in glioma cells. **A,** Time course expression of ATF3, CHOP, and DR5 with NEO214 treatment at 50 μmol/L in U251. **B,** U251 was treated with increasing doses of NEO214, for 20 hours and Western blots were performed for DR5 expression. **C,** Equimolar concentration (100 μmol/L) of each drug or combination of drug were used for treating U251, U251TR, LN229TR, and T98G for 24 hours and Western blot analysis was performed for DR5 expression (C, no treatment control; P, POH; R, Rolipram; R+P, mixture of two drugs; NEO, NEO214; all used 100 μmol/L). **D,** IHC for DR5 after treating with NEO214 (100 μmol/L) for 24 hours. Cells with positive staining for DR5 shows dark red color. **E,** U251 and T98G were treated with different doses of NEO214 with or without TRAIL (10 ng/mL) for 48 hours; cell death was determined using the Alamar blue assay. **F,** Comparison of cell survival using DR5 knockdown U251 (U251-DR5KD) cells by treating NEO214 with or without TRAIL (10 ng/mL) in CFA. U251 and U251-DR5KD cells were treated with NEO214 50 μmol/L for 24 hours; and Western blot analysis was performed for DR5. **G,** U251 tumor cells were treated with NEO214 [N: 50 μmol/L], Trail [T] (5 ng/mL) or combination of NEO214 and TRAIL [N+T]; Western blots were performed evaluating three different pathways. For the intrinsic pathway determination, CHOP, ATF3, and caspase 9 were evaluated. For the extrinsic pathway determination, DR5 and cleaved caspase-8 antibody were evaluated.

Furthermore, DR5 was expressed only when cells were treated with NEO214 and not equimolar concentrations of POH, rolipram, or POH plus rolipram on a variety of resistant glioma cells (Fig. 4C). To identify the distribution of DR5 on NEO214-treated cells, IHC was performed (Fig. 4D). Glioma cells treated with NEO214 (100 μmol/L) for 24 hours demonstrated DR5-positive cells on over 90% of the tumor population. Positive staining was identified as a red precipitate; tumor cells not treated with

NEO214 exhibited no staining. NEO214-induced DR5 was expressed on all glioma cell lines tested. To determine whether DR5 induced by NEO214 was functional, NEO214-activated cells were treated with TRAIL, the ligand which binds to DR5 and induces cell death (40). Glioma cells were treated with NEO214 in the absence or presence of TRAIL (10 ng/mL) for 48 hours. As shown in Fig. 4E, NEO214 caused cytotoxicity as expected; however, the addition of TRAIL to NEO214 caused a dramatic increase in cytotoxicity in both temozolomide-sensitive (U251) and temozolomide-resistant (T98G) gliomas. These data show that NEO214 can induce cytotoxicity through the TRAIL/DR5 pathway, as well as through ER stress. To determine the extent to which DR5 expression plays a role in NEO214-mediated cytotoxicity, we knocked down DR5 using lentiviral shRNA-DR5 (Fig. 4F). The Western blot data demonstrate the efficiency of DR5-KD is more than 50%. The cytotoxicity results showing that the combination treatment of NEO214 and TRAIL with knockdown (KD) strains did not show a significant decrease in cell survival (Fig. 4F), suggesting that the increased cytotoxicity observed with TRAIL in Fig. 4E was the result of DR5 expression. We treated glioma cells with NEO214 only, TRAIL only, and NEO214 combined with TRAIL, and performed Western blot analysis to confirm the pathways that are involved in this treatment. As shown in Fig. 4G, both intrinsic and extrinsic pathways of apoptosis were activated with NEO214 and TRAIL treatment. Caspase 9 cleavage is indicative of intrinsic pathway involvement and caspase 8 cleavage is suggestive of the extrinsic pathway activation. Thus, both cytotoxic pathways are activated. Therefore, we demonstrated here that the addition of TRAIL enhances the cytotoxic effects of NEO214 by activating both the intrinsic and extrinsic apoptotic pathways.

NEO214 is not cytotoxic to normal cells

To determine whether NEO214 is cytotoxic for nonmalignant cells, primary cultures of normal human astrocytes and human brain-derived endothelial cells (BEC; Fig. 5A) were treated with NEO214 for 3 days, and evaluated for cytotoxicity. The results showed that NEO214 was not cytotoxic for these normal cell populations at the dose range of NEO214 tested (0–200 μmol/L). To determine whether TRAIL alone enhanced cell death in normal cells, NEO214 was tested in the absence or presence of TRAIL (10 ng/mL). As shown in Fig. 5B, this combination had no significant effect on survival of astrocyte at NEO214 doses ranging from 0 to 100 μmol/L. We treated astrocytes with NEO214 (100 μmol/L), and analyzed these cells for ER stress markers, apoptosis markers, and DRs. Glioma cells treated with NEO214 (50 μmol/L) was the positive control (PC). The results demonstrated that astrocytes do not express CHOP, and are weakly active for ATF3 (Fig. 5C). At this dose, NEO214 did not cause cell death in astrocytes, as shown by the lack of cleaved PARP (Fig. 5C). DR5 expression in astrocytes (Fig. 5D) was examined to confirm the lack of DR5 expression in astrocytes. IHC in the absence or presence of NEO214 showed no detectable DR5 expressed in astrocytes (Fig. 5E). TRAIL was, however, constitutively expressed by astrocytes as confirmed by Western blot analysis (Fig. 5D) and TRAIL ELISA (Fig. 5F). Astrocytes constitutively secrete functional TRAIL in the absence or presence of NEO214, as confirmed by the Alamar blue assay with NEO214 only or NEO214 with astrocyte culture supernatant (SUP) or astrocyte cell lysates (Lys). The results confirmed that astrocytes secrete functional TRAIL resulting in tumor cell death in combination with NEO214 (Fig. 5G). These data also confirm that NEO214 is not cytotoxic to astrocytes because DR5 expression is required for cell death and astrocytes lack DR4/5 expression.

NEO214 activates tumor cell death in 3D coculture of tumor cells and astrocytes

To analyze the cell interactions leading to apoptosis, glioma cells were labeled with GFP, seeded on coverslips, and cultured for 24 hours. The next day, cells were treated with NEO214 for 24 hours. Figure 6A showed the confocal image of the control, no drug–treated GFP-U251 cells (U251-GFP: left box), and NEO214-treated GFP-U251 cells (U251-GFP [NEO214 50 μmol/L]: right box). The blue nuclear DAPI staining identified total numbers of cells showing that NEO214 (50 μmol/L) treated for 24 hour did not cause tumor cell death. The coculture of tumor cells and astrocytes were performed by seeding astrocytes onto coverslips first and incubating for 24 hours. Subsequently, GFP-U251 cells were seeded on top of the astrocytes and cultured for another 24 hours. At this point, cocultured cells were treated with or without 50 μmol/L of NEO214 for another 24 hours. After 24-hour incubation, coverslips with cocultured cells were fixed and immunostained. GFP (green)-positive tumor cells, red astrocytes stained for glial fibrillary acidic protein (GFAP), and blue nuclear DAPI staining were analyzed (Fig. 6B). Cocultures without NEO214 treatment (glioma GFP/Astrocyte: left box) showed that both GFP-positive tumor cells (green) and astrocytes (red) remained intact. However, the confocal image of NEO214-treated cocultures of astrocytes and tumor cells demonstrated extensive death of the green tumor cells [glioma-GFP/Astrocyte (NEO214 50 μmol/L): right box]. These data indicate that optimal tumor cell death requires NEO214 to upregulate DR5 expression on tumor cells. Nonmalignant cells are not induced to express DR4/5; therefore, these cells are not affected by NEO214. These data show that cell death is specific for tumor cells expressing the death receptor. *In vitro*, TRAIL was added to the cell cultures together with NEO214 for maximum cytotoxicity. *In vivo*, in the brain microenvironment, TRAIL is provided by the astrocytes. Thus, *in vivo*, the brain provides the ideal microenvironment for activation of the DR5-induced apoptotic pathway.

NEO214 decreases glioma progression in the *in vivo* mouse tumor model

To determine whether NEO214 is effective in the brain tumor progression *in vivo*, we used two different animal models: (i) intracranial athymic nude mouse bearing human tumor cells, given subcutaneously; (ii) intracranial immune competent syngeneic (C57bl/6) mouse. For the athymic nude mouse model, luciferase-labeled human glioma cells were implanted intracranially. Animals were imaged every other week and survival was monitored. As shown in Fig. 7A, animals treated subcutaneously with NEO214 (50 mg/kg) demonstrated a significant decrease in tumor growth as compared with vehicle control group ($P < 0.0032$). The median survival for the vehicle group was 27 days, while the median survival for NEO214-treated group was 78 days; the HR (log-rank) was 6.77. There was a 189 % increase in survival time with NEO214 treatment. These data also indicated that NEO214 is able to cross the BBB. To test the syngeneic immunocompetent model, GL261 mouse glioma cells were implanted intracranially into C57bl/6. From previous experience, tumor take of GL261 in C57bl/6 was 100 %. Animals were randomly grouped ($n = 4$): (i) untreated, (ii) vehicle- treated, (iii) NEO214 (100 mg/kg) treated subcutaneously, or (iv) NEO214 oral gavage administration.

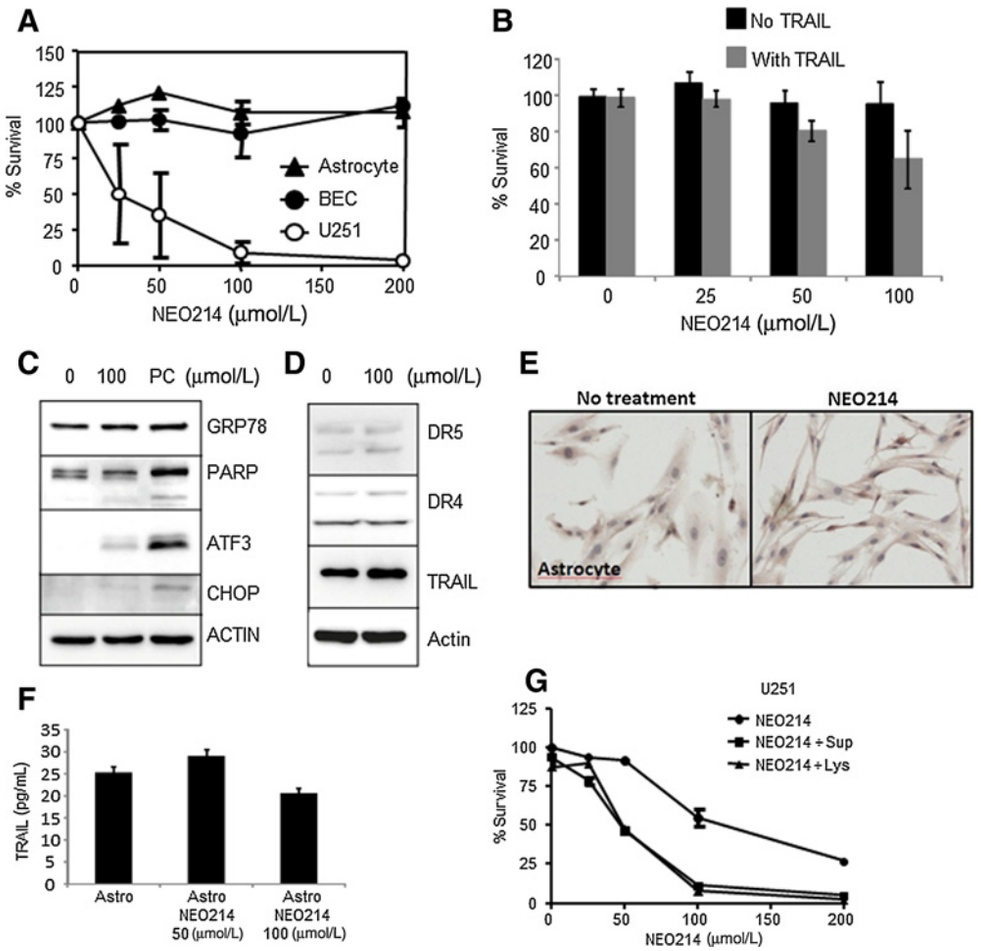

Figure 5.

NEO214 and its combination with TRAIL is not cytotoxic to astrocytes. **A,** Normal human astrocytes and human brain endothelial cells (BEC) were incubated with NEO214 for 3 days and survival was measured using Alamar blue assay. Cell survival was expressed as percentage survival compared with untreated cells. **B,** Astrocytes were treated with different concentrations of NEO214 in the absence or presence of TRAIL (10 ng/mL), for 48 hours, and cell survival was analyzed by Alamar blue assay. **C,** Astrocytes were treated with NEO214 100 μmol/L for 24 hours; and Western blots for GRP78, CHOP, ATF3, and PARP were performed (PC, positive control). **D,** Astrocytes were treated with 100 μmol/L of NEO214 for 24 hours, and then tested for DR5, DR4, and TRAIL. **E,** IHC for DR5 was performed using astrocytes treated with NEO214 100 μmol/L for 24 hours. **F,** Supernatants derived from cultures of astrocytes with or without NEO214 were analyzed for TRAIL secretion with Human TRAIL Immunoassay Kit. **G,** Cytotoxic assay of NEO214 in U251 with or without astrocyte supernatants (SUP) or astrocyte lysates (Lys).

NEO214 was administered on a schedule of 5 days on and 2 days off. This dosing schedule was decided on the basis of our previous experimental results. Animal survival was monitored. As shown in Fig. 7B, the median survival for the untreated group was 21.5 days and for the vehicle group was 22 days. The median survival for the NEO214-treated group by subcutaneous (s.c.) delivery was 29 days ($P = 0.01$), and oral gavage administration of NEO214 was 28.5 days ($P = 0.03$). Both subcutaneous and oral administration of NEO214 increased survival. Thus, there was approximately 35% increase in survival time with NEO214 treatment in the syngeneic mouse model; this difference was independent of mode of administration. These data also indicate that NEO214 crosses the BBB. To determine whether NEO214 stimulated CHOP and DR5 in this immunocompetent brain tumor model, we implanted GL261 mouse glioma cells into the syngeneic mouse frontal cortex; 10 days postimplantation, the mice were treated with NEO214 (100 mg/kg) subcutaneously. Mouse brains were harvested after 24, 48, and 72 hours, and stained for Ki67, CHOP, and DR5. As shown in Fig. 7C, Ki67 staining (proliferation marker) was positive in the tumor area (black arrows). Furthermore, we found that NEO214 induced CHOP and DR5 in the brain tumors. CHOP was expressed within 24 hours of NEO214 administration, and DR5 was expressed after 48 hours. For the toxicity evaluation, to determine whether long-term treatment (20 days daily) of NEO214 (100 mg/kg) was toxic, we evaluated the H&E staining of spleens and bone marrows with and without NEO214 treatments (Fig. 7D). NEO214 treatment showed no toxicity at the dose tested.

Discussion

This study demonstrates the antitumor efficacy of NEO214, a conjugate of rolipram and perillyl alcohol. We have shown that

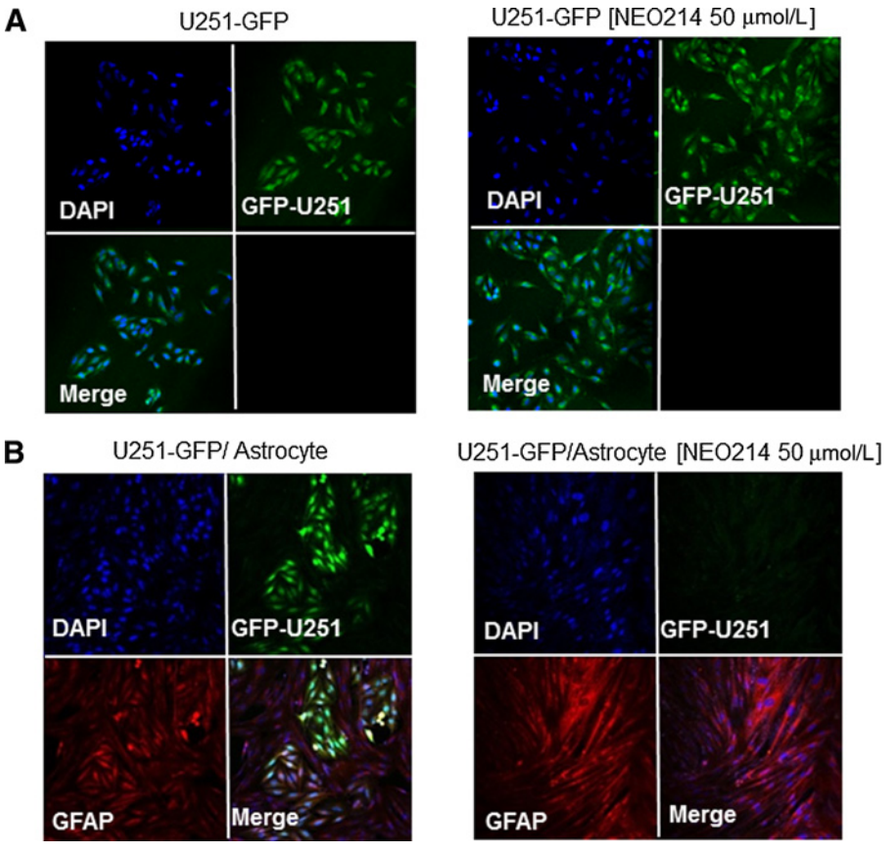

Figure 6.
3D Coculture of astrocyte and GFP-U251. **A,** Confocal image of GFP-U251 (GFP: green) without NEO214 (left) and with NEO214 50 μmol/L treatment (right) for 24 hours. DAPI staining for nucleus (blue). **B,** Confocal image of GFP-U251 (GFP: green) and astrocyte (GFAP: red) coculture without NEO214 treatment (left) and with NEO214 treatment for 24 hours (right).

the activity of NEO214 is not attributed to rolipram or POH alone, or the mixture of rolipram and POH, but to the covalently bound molecules, rolipram and POH. This unique drug maintains its cytotoxic capacity up to 48 hours in media at 37°C. The half-life of NEO214 in serum and in blood was 4–6 hours (Supplementary Fig. S2A and S2B). This information suggests that NEO214 is a relatively stable drug and may be administered less often than other drugs. NEO214 is effective *in vivo* by causing glioma cytotoxicity resulting in reduced GBM progression. Rolipram, is a selective inhibitor of phosphodiesterase 4 (PDE4), and as a result, increases intracellular cAMP levels, which has been reported to have antitumor activity (5, 6). Our studies have shown that NEO214 can inhibit PDE4, and cause a low level increase in cAMP in the tumor cells (41). To determine the effects of increased intracellular cAMP on antitumor activity, we combined NEO214 and forskolin, an agent that elevates cAMP by activating adenylyl cyclase (42). The results showed that upregulated cAMP does not increase cytotoxic activity of tumor cells, suggesting that mechanisms other than modulating cAMP are responsible for the observed antitumor effects of NEO214 (41). In acute toxicity studies, rolipram alone at doses of 10 mg/kg resulted in seizure and subsequent recovery within 24 hours of administration. In contrast, NEO214 administered at doses as high as 200 mg/kg did not cause any behavioral or histologic changes as compared with vehicle (data not shown). These data support the idea that NEO214 functions differently as compared with rolipram.

Our studies show that NEO214 induces cytotoxicity in glioma cells and patient-derived glioma cells that vary with respect to mechanisms of temozolomide resistance. The temozolomide-resistant glioma cell lines, U251 TR and LN229 TR, were prepared by continuous exposure of glioma cells to temozolomide (22). Glioma cells (U251 TR) were shown to be temozolomide-resistant as a result of overexpression of the base excision repair (BER) pathway, while the temozolomide resistance in LN229TR glioma cells was the result of deficiency of the specific mismatch repair proteins (22). T98G glioma cells overexpress the DNA repair protein, MGMT (43), which is resistant to temozolomide but sensitive to NEO214. Patient-derived glioma cells USC02 is MGMT-positive mesenchymal type, USC04 is MGMT-negative proneural type, and USC10 is MGMT-negative mesenchymal type. Patient-derived glioma cells are more resistant to NEO214 compared with human glioma cells. This difference may be due to the enriched glioma stem cells, as a result of the fact that patient-derived cells are cultured in stem cell media, which expands the stem cell population. These stem cells are known to be more resistant than non-stem cell tumor populations (44). Thus, NEO214 is cytotoxic for temozolomide-resistant glioma cells, derived from different mechanisms of temozolomide resistance. NEO214 is cytotoxic at relatively low doses, without any apparent negative effects on normal cells such as human BEC and human astrocytes.

Understanding the various cytotoxic mechanisms of this drug will be useful for developing appropriate combination therapies. Our studies show that NEO214 triggers tumor cell death using the ER stress pathway. ER stress can be the result of the accumulation of unfolded protein response (UPR) resulting in the disruption of Ca^{2+} homeostasis in ER. Our results show that NEO214 does not

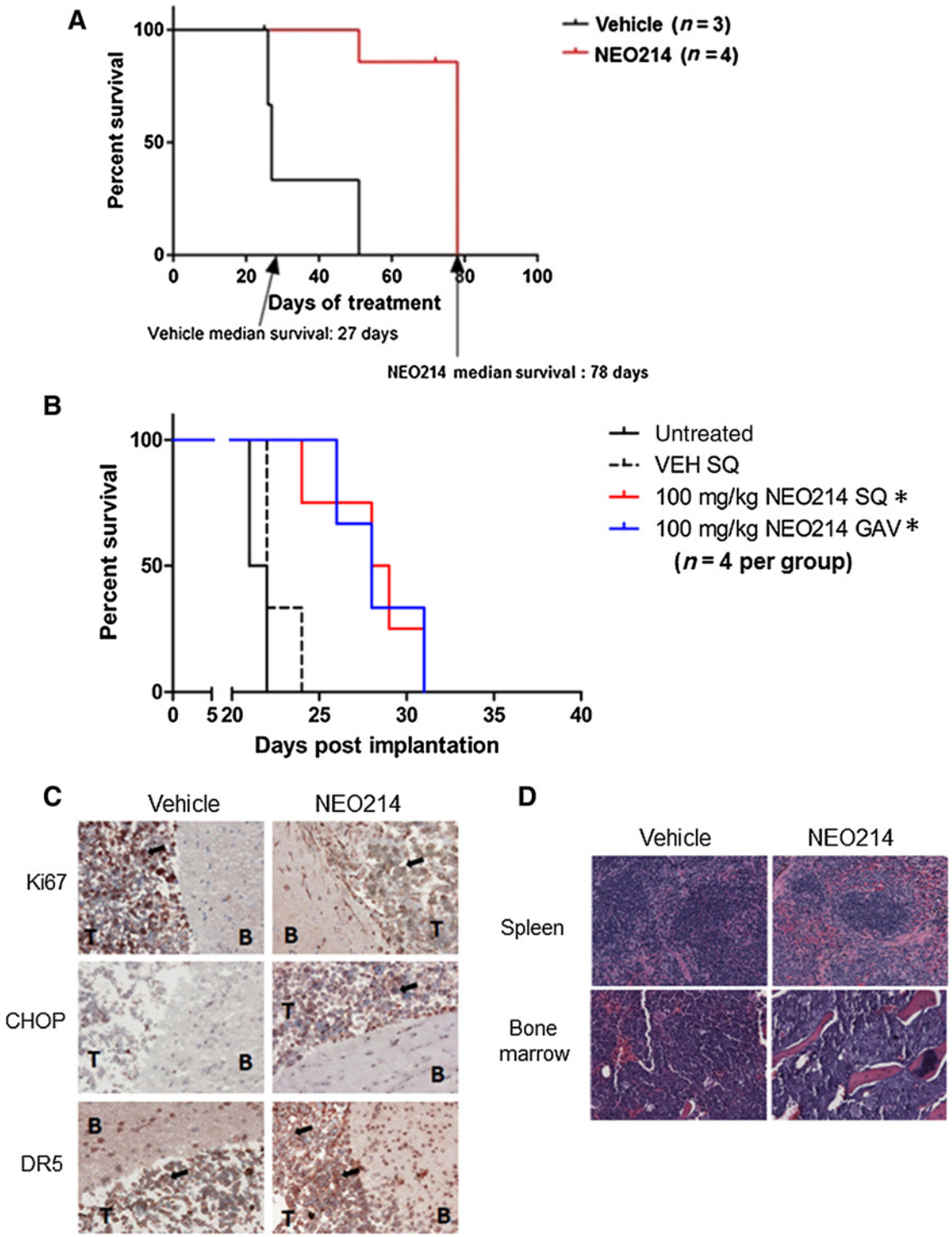

Figure 7.

NEO214 has antitumor effects *in vivo* syngeneic intracranial mouse model. **A,** Luciferase-positive human glioma cells (U251) were intracranially injected in the frontal lobe of the athymic nude mice. NEO214 (50 mg/kg) was administered subcutaneously for 30 days with a 5 days on and 2 days off schedule. Tumor growth was monitored by imaging the mice and survival was monitored. **B,** GL261 mouse glioma cells (1×10^5) were intracranially injected in the frontal lobe of the C57bl/6 mice. NEO214 (100 mg/kg) was administered subcutaneously with a 5 days on and 2 days off schedule for 30 days. The mice survival was monitored. **C,** Ki67, CHOP, and DR5 staining in the GL261 implanted C57bl/6 mouse brain tumor 24, 48, and 72 hours after treatment of NEO214. Black arrow shows representative positive staining. T, tumor area; B, brain area (**D**). Specimens were evaluated for pathologic differences between untreated and NEO214-treated animals. Spleens and bone marrows from C57bl/6 mice that were treated with vehicle and NEO214 (100 mg/kg) for 20 days were analyzed.

inhibit SERCA; however, NEO214 does cause the release of free Ca^{2+} into the cytoplasm, resulting in the induction of CHOP and cell death. The combination of NEO214 with anisomycin blocks the Ca^{2+} release in the cytoplasm revealing that NEO214 may be acting as translocon opener similar to puromycin; this function is currently under investigation with NEO214 as a potential translocon opener. CHOP is a critical apoptotic protein and key to ER stress–mediated cell death. Our data show that reducing CHOP expression increases cell survival (Fig. 3H), suggesting that NEO214-induced cytotoxicity is mediated through the ER stress pathway. However, other cytotoxicity pathways may be relevant, as shown by only a 30% rescue with reduced CHOP. Our studies demonstrate that NEO214 can also induce cell death through activation of the DR5/TRAIL pathway. NEO214 induces DR5 expression in all glioma cell populations that we have tested (e.g., U251, LN229, T98G). Western blot analyses showed that the combination of NEO214 and TRAIL induced the activation of caspase 8, a cytotoxic mediator for the extrinsic pathway. Knockdown of DR5 eliminated cell death in the presence of TRAIL. The combination of DR5 and TRAIL demonstrated how critical are the cellular interactions of these proteins, particularly in the brain microenvironment. Here, we show that astrocytes secrete TRAIL while NEO214 induces DR5 expression on tumor cells. Analysis of astrocytes cultures demonstrate that these cells constitutively secrete functional TRAIL as determined by 3D cocultures of tumor cells with astrocytes. We have observed that cocultures of tumor cells with astrocytes, in the absence of NEO214, did not induce tumor cell death. However, treatment with NEO214 in 3D cocultures caused dramatic tumor cell death indicating that NEO214 stimulates DR5 on tumor cells, and the astrocytes contribute TRAIL. It should be noted that DR5 cannot be induced on astrocytes. Only cells that express DR5 will undergo cytotoxicity through this pathway. Thus, NEO214 is especially effective in the central nervous system environment. Our *in vitro* 3D coculture data showed that the induction of DR5 in tumor cells with NEO214, and the presence of TRAIL caused effective and specific tumor cell death. Therefore, the data show that astrocytes were responsible for selective tumor cell death in the brain microenvironments. These data show that the brain parenchyma provides the optimal environment for activating NEO214 cytotoxicity by selectively stimulating DR5 expression in tumor cells by NEO214. Previously published immunochemistry data with various human brain tumors showed that TRAIL expression was observed in areas with a significant mixture of astrocytic cells and the pattern of TRAIL expression. We also stained human GBM tissue specimens for GFAP and TRAIL and found constitutive expression of TRAIL associated with astrocytes in double staining (Supplementary Fig. S1).

Our studies show that NEO214 is effective in reducing glioma progression *in vivo* confirmed by using intracranial nude mice model and intracranial syngeneic C57bl/6 mouse model. The dose of NEO214 was chosen based on our preliminary toxicity studies with 50 and 100 mg/kg. We found no evidence of toxicity in mice treated daily for 20 days. Therefore we choose 50 mg/kg and 100 mg/kg for *in vivo* studies. We have not measured the NEO214 concentrations in the brain tumor area, but have shown in Fig. 7C, a one-time treatment of 100 mg/kg of NEO214 was enough to induce ER stress in the mouse brain tumors. After treating the mouse once with 100 mg/kg of NEO214, we found CHOP expression after 24 hours and DR5 expression after 48 hours. Therefore, in the *in vivo* environment, 100 mg/kg dose was sufficient to induce ER stress in the brain environment. There was no difference in the survival pattern between subcutaneous and gavage administration, most likely due to the relatively long stability of the drug. Therefore, drug administration protocol may not be critical for NEO214. The histologic analysis of spleen and bone marrows, exhibited no observable differences between the vehicle-treated group and NEO214-treated group (Fig. 7D). We also collected blood samples from these mice and analyzed the white blood cells, red blood cells, and platelets (data not shown); there were no apparent differences in numbers compared with normal tissue. We performed MTD experiments and found that up to 400 mg/kg, no signs of acute and chronic toxicity were detected in any of the organs and blood cell counts (data not shown). NEO214, the doses we tested were not toxic to the animals. We are currently investigating additional doses.

The specificity of NEO214 lies in its ability to selectively induce DR5 on tumor cells, and not on normal brain tissue. Its ability to utilize normal astrocytes and their secreted TRAIL ligands is potentially a novel treatment for all infiltrating cancers. Because primary gliomas infiltrate into the normal white matter, this drug can potentially reduce the number of tumor cells in the microenvironment or prevent further invasion into the white matter. The different potential activities of NEO214 using human-patient derived glioma cells are currently being examined. The ability of NEO214 to induce DR5 in other primary brain tumors (i.e., meningiomas, pituitary adenomas) and metastatic brain cancer are under investigation. In summary, our data demonstrate that NEO214 is a novel drug that has minimal toxicity for normal cells and effectively enters the brain. These findings show that NEO214 has great potential as a therapeutic agent for the treatment of newly diagnosed and recurrent GBM, and can provide long-term clinical benefit for patients with cancer.

Disclosure of Potential Conflicts of Interest

Dr. Thomas C. Chen is an officer and shareholder of NeOnc Technologies, Inc. The other authors do not disclose any conflict of interest.

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H.-Y. Cho, S.D. Swenson
Study supervision: H.-Y. Cho

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