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Apoptotic and antiproliferative effects of amantadine and rimantadine in glioblastoma cells

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ABSTRACT

Introduction: Glioblastoma (GBM) is a highly invasive tumor, which exhibits a poor response to standard chemotherapeutic agents. Amantadine, an anti-influenza A with anti-Parkinson's effect, inhibits cell proliferation and induces apoptosis in hepatocellular cancer cells. Rimantadine, a derivative of amantadine with comparable effect on influenza, also improves symptoms of Parkinson's disease. **Objectives:** The present study aimed to investigate apoptotic and antiproliferative effects of amantadine and rimantadine in two GBM cell lines, A172 and U-87 MG. **Materials and Methods:** Cell viability was measured by methyl thiazolyl tetrazolium assays. Cell apoptosis, cell proliferation, and cell cycle analysis were determined by flow cytometry using Annexin V/7-aminoactinomycin D, carboxyfluorescein diacetate succinimidyl ester, and propidium iodide with RNase staining, respectively. **Results:** Rimantadine demonstrated more cytotoxic effect than amantadine in both GBM cell lines. Rimantadine at 500 μM increased the percentage of early and late apoptosis of GBM cells, while amantadine at the same concentration induced the late apoptosis only in U-87 MG cells. Amantadine and rimantadine at 250–500 μM suppressed cell proliferation only in U-87 MG cells. Furthermore, 500 μM of both drugs induced G0/G1 arrest in U-87 MG cells. **Conclusion:** Amantadine and rimantadine induce apoptosis and inhibit proliferation through G0/G1 arrest; however, rimantadine shows higher toxicity, suggesting the greater therapeutic potential of rimantadine for GBM.

Keywords: Amantadine, apoptosis, cell cycle arrest, glioblastoma cells, rimantadine

INTRODUCTION

Amantadine and rimantadine (the α-methyl derivative of amantadine) are used for the treatment of influenza A. Both drugs block M2 proton channels of the virus particle and inhibit the uncoating process of viral RNA, thus viral replication is prohibited.[1] Amantadine is also approved as an adjunctive treatment for Parkinson's disease. This drug inhibits N-methyl D-aspartate receptors and increases dopamine transmission.[2,3] However, the use of rimantadine in Parkinson's disease is limited only in pilot studies and case reports. Rimantadine has similar neuropharmacological properties as amantadine and provides improvement of motor symptoms in patients with Parkinson's disease with mild and transient side effects.[4,5]

Amantadine and rimantadine are equally efficacious in preventing influenza A.[6] Central nervous system adverse effects and study withdrawal are more common with amantadine than rimantadine in healthy volunteers[7] and elderly nursing home patients.[8] The higher rates of confusion and agitation are reported with amantadine use. Amantadine at doses 30 and 60 mg/kg for 3 days increases DNA damage in brain tissues and produces locomotor disturbances in adult male mice, suggesting its neurotoxicity.[9]

Glioblastoma (GBM) is the most common malignant primary tumor of the brain involving glial cells. It is the most aggressive manifestation of malignant gliomas. GBM has deregulation of cell cycle progression, highly proliferative capacity, and resistance to apoptosis.[10,11] Amantadine shows *in vitro* anticancer effect by suppressing proliferation and inducing apoptosis in hepatocellular cancer cells.[12] However, the anticancer effects of amantadine and rimantadine on human GBM cells have not been elucidated. Herein, apoptotic and antiproliferative effects of amantadine and rimantadine were tested on A172 and U-87 MG human GBM cells.

MATERIALS AND METHODS

Drugs

Amantadine hydrochloride and rimantadine hydrochloride were purchased from Sigma-Aldrich (St Louis, MO, USA) and dissolved in sterile distilled water at a stock concentration of 0.1 M and stored at −20°C until use for the maximum of 2 weeks.

Cell Culture

The human GBM cell lines, A172 and U-87 MG, were obtained from American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (DMEM) and minimal essential medium (MEM) containing 1% sodium pyruvate, respectively. Both cells were supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Invitrogen). Cells were incubated at 37°C in a humidified 5% CO_2/air atmosphere.

Methyl Thiazolyl Tetrazolium (MTT) Cell Viability Assay

A172 and U-87 MG cells were plated in 96-well plates at a density of 8000 and 15,000 cells/well, respectively. After reaching 70% confluence, various concentrations (1–1000 μM) of amantadine or rimantadine were added, and cultivation of the cells was continued for 24 and 48 h. The effect on cytotoxicity was determined by MTT assays. Briefly, 50 μl MTT solution (5 mg/ml) was added to each well, and the cells were incubated for another 2 h at 37°C. Subsequently, 60 μl dimethyl sulfoxide was added to dissolve the MTT formazan, and the absorbance was measured using a microplate reader at a wavelength of 562 nm.

Annexin V/7-aminoactinomycin D (7-AAD) Apoptosis Assay

Cells were plated in 6-well plate until 70% confluency and treated with 500 μM of amantadine or rimantadine for 24 and 48 h. The cell suspension was labeled with fluorescein isothiocyanate (FITC)-conjugated Annexin V (BD Biosciences, San Jose, CA, USA) and 7-AAD (BD Biosciences, San Jose, CA, USA) staining as previously described.[13] Cells were analyzed by a BD Accuri C6 flow cytometer (BD Biosciences), and the percentage of apoptotic cells was evaluated.

Carboxy-fluorescein Diacetate N-succinimidyl Ester (CSFE) Cell Proliferation Assay

Cells were labeled with 1 μM CSFE (Sigma-Aldrich, St Louis, MO, USA) as previously described.[13] After washing the excess CSFE, cells were seeded in media containing 5% FBS in 6-well plates and treated daily with amantadine or rimantadine at 125–500 μM for 48 and 72 h. Fluorescence intensity of living cells was measured by a BD Accuri C6 flow cytometer. Mean fluorescence intensity (MFI) of each treatment was compared to MFI of mock-treated cells at each time point.

Cell Cycle Analysis

U-87 MG cells were placed in 6-well plates and cultured for 24 h with 500 μM amantadine or rimantadine. Cells were fixed in 70% ice-cold ethanol and stained with propidium iodide and 0.5 mg/ml RNase (BD Biosciences, San Jose, CA, USA) as previously described.[13] Fluorescence intensity was analyzed by a BD Accuri C6 flow cytometer (BD Biosciences).

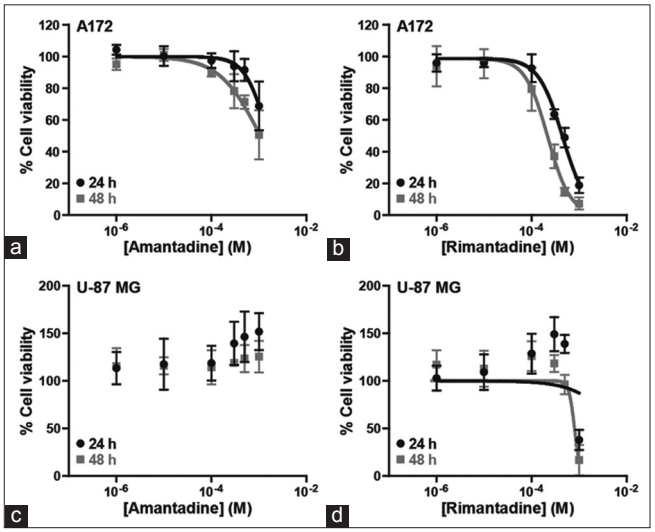

Figure 1: Cytotoxicity of amantadine and rimantadine in glioblastoma (GBM) cell lines. A172 and U-87 MG GBM cells were treated with amantadine or rimantadine at 1–1000 μM for 24 and 48 h. Cell viability of GBM cells was measured by methyl thiazolyl tetrazolium assays. (a) Amantadine and (b) rimantadine inhibited cell survival in A172 cells in a dose- and time-dependent manner. (c) Amantadine showed no cytotoxicity to U-87 MG cells at 24 and 48 h. (d) Rimantadine at 1000 μM reduced U-87 MG cell viability at 24 and 48 h. Each dot represented the average (± SD) of the percentage of viable cells. TC_{50} values were estimated from at least three independent experiments using concentration-inhibition curves by non-linear curve fitting (GraphPad Prism)

Table 1: Median toxic concentrations of amantadine and rimantadine in A172 and U-87 MG cells

Cell type	TC_{50} values	
	Amantadine	Rimantadine
A172		
24 h	>1000 μM	452.3 μM
48 h	>1000 μM	219.6 μM
U-87 MG		
24 h	ND	978.1 μM
48 h	ND	803.1 μM

TC_{50}: The 50% toxic concentration, ND: Not determined

Statistical Analysis

All data were presented as mean ± standard deviation (SD). One-way analysis of variance followed by Dunnett's multiple comparison test was analyzed the statistical significance between the amantadine or rimantadine-treated group and mock-treated group using GraphPad Prism version 7.0 (GraphPad Software Inc., La Jolla, CA, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

Amantadine and Rimantadine Decreased Cell Viability of GBM Cells

The effect of amantadine and rimantadine on growth and viability of GBM cells was determined by MTT assays. The median toxic concentrations (TC_{50}) of amantadine in A172 cells at 24 and 48 h were >1000 μM at both time points [Figure 1a, Table 1]. In contrast, the TC_{50} values of rimantadine at 24 and 48 h were 452.3 and 219.6 μM, respectively [Figure 1b, Table 1]. Rimantadine at 1000 μM reduced A172 cell viability to <20% and 10% at 24 and 48 h post-exposure, respectively, while cells exposed to amantadine had viability about 50% at the same concentration. In U-87 MG cells, amantadine at concentration 300–1000 μM slightly increased cell viability at 24 h, while there was no change in cell viability at 48 h [Figure 1c]. The TC_{50} values of rimantadine in U-87 MG cells at 24 and 48 h were 978.1 and 803.1 μM, respectively [Figure 1d, Table 1].

Amantadine and Rimantadine Induced Apoptosis of GBM Cells

To test whether amantadine or rimantadine reduced cell viability by induction of apoptosis or necrosis, GBM cell lines were treated with 500 μM of amantadine or rimantadine for 24 and 48 h, and then the percentage of apoptotic cells was evaluated by Annexin V and 7-AAD staining. Non-apoptotic viable cells showed negative staining with Annexin V and 7-AAD. In the early stage of apoptosis, cells were stained with Annexin V without 7-AAD staining while cells in the later stage of apoptosis showed positive staining for both Annexin V and 7-AAD. After 24 h exposure, rimantadine increased late apoptotic cells in A172 cells compared to mock-treated cells (13.2% vs. 6.9%, $P < 0.01$) [Figure 2a and b]. After 48 h post-exposure, rimantadine increased levels of early and late apoptotic cells of A172 cells ($P < 0.05$) [Figure 2a and c]. In contrast, amantadine did not induce apoptosis of A172 cells at 24 and 48 h post-exposure [Figure 2].

In U-87 MG cells, rimantadine increased early apoptotic cells from 3.4% to 11.9% after 24 h ($P < 0.05$) [Figure 3a and b]. At 48 h post-exposure of rimantadine induced both early and late apoptotic cells from 1.2 to 6.9% ($P < 0.05$) and from 2.8 to 13.5% ($P < 0.01$), respectively [Figure 3b and c]. Amantadine was also increased late apoptotic cells to 6% ($P < 0.05$) [Figure 3b and c].

Amantadine and Rimantadine Inhibited Cell Proliferation of U-87 MG Cells

We further investigated the antiproliferative effect of amantadine and rimantadine by carboxyfluorescein diacetate succinimidyl ester (CFSE) proliferation assays using flow cytometry. The MFI

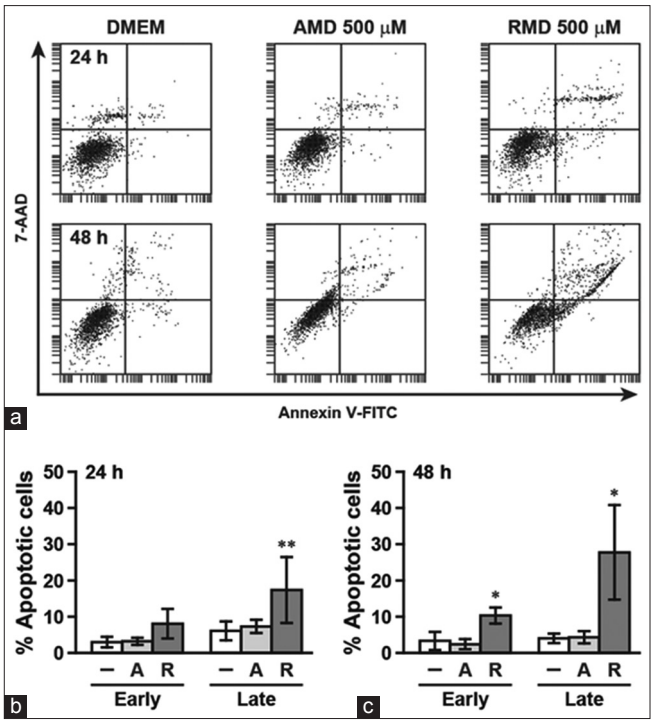

Figure 2: Induction of apoptosis of A172 cells by amantadine and rimantadine. The percentage of apoptotic cells was determined using flow cytometry. (a) Representative scatter plots demonstrated A172 cells in the early stage of apoptosis showed Annexin V-positive and 7-aminoactinomycin D (7-AAD)-negative and cells in the late stage of apoptosis showed Annexin V-positive and 7-AAD-positive staining after 500 μM amantadine or rimantadine exposure at 24 (top) and 48 h (bottom). (b and c) Bar graphs showed the mean percentage (± SD) of early and late apoptosis in A172 cells after 500 μM amantadine and rimantadine exposure at 24 (left) and 48 h (right). All data were representative of at least three independent experiments. One-way ANOVA with Dunnett's multiple comparison test was used for statistical analysis; *$P < 0.05$ and **$P < 0.01$. AMD and A; amantadine; 7-AAD: 7-aminoactinomycin D; DMEM: Dulbecco's modified Eagle's medium; FITC: Fluorescein isothiocyanate; RMD and R: Rimantadine

is reduced after each cell division; therefore, the lower MFI indicates greater cell proliferation. At 72 h, a rightward shift of fluorescence intensity was observed in U-87 MG cells treated with amantadine [Figure 4a] and rimantadine [Figure 4b]. At 48 h, only rimantadine at concentration 500 μM increased the MFI compared to mock-treated cells ($P < 0.05$) [Figure 4c, left]. After 72 h of treatment, both amantadine and rimantadine caused a significant increase in the MFI of U-87 MG cells at concentration 250 μM ($P < 0.05$ and $P < 0.01$, respectively) and 500 μM ($P < 0.01$) [Figure 4c, right]. In contrast, amantadine and rimantadine did not affect cell proliferation in A172 cells at any concentration and time [Figure 4d].

Amantadine and Rimantadine Induced G0/G1 Phase Arrest in U-87 MG Cells

To explore how amantadine and rimantadine inhibited proliferation of U-87 MG cells, cell cycle distribution was assessed by flow cytometry at 24 h. Amantadine and rimantadine at 250 and 500 μM increased the percentage of cells in the G0/G1 phase ($P < 0.01$) with a concomitant reduction in the S and G2/M phases [Table 2].

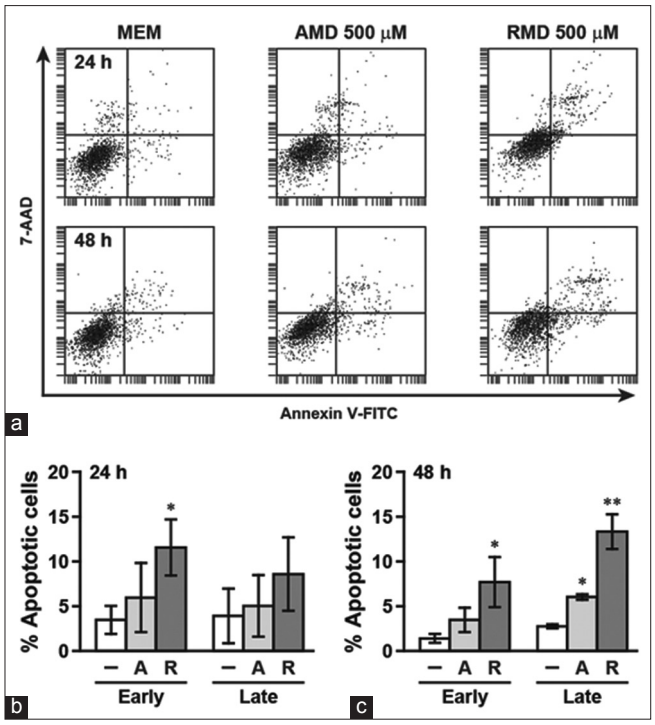

Figure 3: Induction of apoptosis of U-87 MG cells by amantadine and rimantadine. The percentage of apoptotic cells was determined using flow cytometry. (a) Representative scatter plots demonstrated U-87 MG cells in the early stage and late stage of apoptosis after 500 μM amantadine and rimantadine exposure at 24 (top) and 48 h (bottom). (b and c) Bar graphs showed the mean percentage (± SD) of early and late apoptosis in U-87 MG cells after 500 μM amantadine and rimantadine exposure at 24 (left) and 48 h (right). All data were representative of at least three independent experiments. One-way ANOVA with Dunnett's multiple comparison test was used for statistical analysis; *P < 0.05 and **P < 0.01. AMD and A: Amantadine; 7-AAD: 7-aminoactinomycin D; FITC: Fluorescein isothiocyanate; MEM: Minimal essential medium; RMD and R: Rimantadine

Table 2: Cell cycle phase distribution of U-87 MG cells treated with amantadine or rimantadine for 24 h

Treatment	Cell cycle progression (%)		
	G0/G1 phase	S phase	G2/M phase
U-87 MG			
MEM	68.8±4.0	9.8±1.7	21.4±2.7
Amantadine 250 μM	72.2±4.0**	7.8±1.5*	19.9±3.1
Amantadine 500 μM	76.9±5.8**	5.6±1.5**	17.5±4.4*
Rimantadine 250 μM	75.1±3.6**	7.2±1.2*	17.7±2.6*
Rimantadine 500 μM	78.3±5.3**	4.3±0.7**	17.3±4.7*

Values were the mean percentage (±SD) of the cell population of at least three independent experiments. One-way ANOVA with Dunnett's multiple comparison test was used for statistical analysis; *P<0.05 and **P<0.01. MEM: Minimal essential medium

DISCUSSION

GBM is the most common malignant primary tumor of the brain involving glial cells. It is the most aggressive manifestation of malignant gliomas with the deregulation of cell cycle progression, highly proliferation, and resistance to apoptosis.[10,11] The present

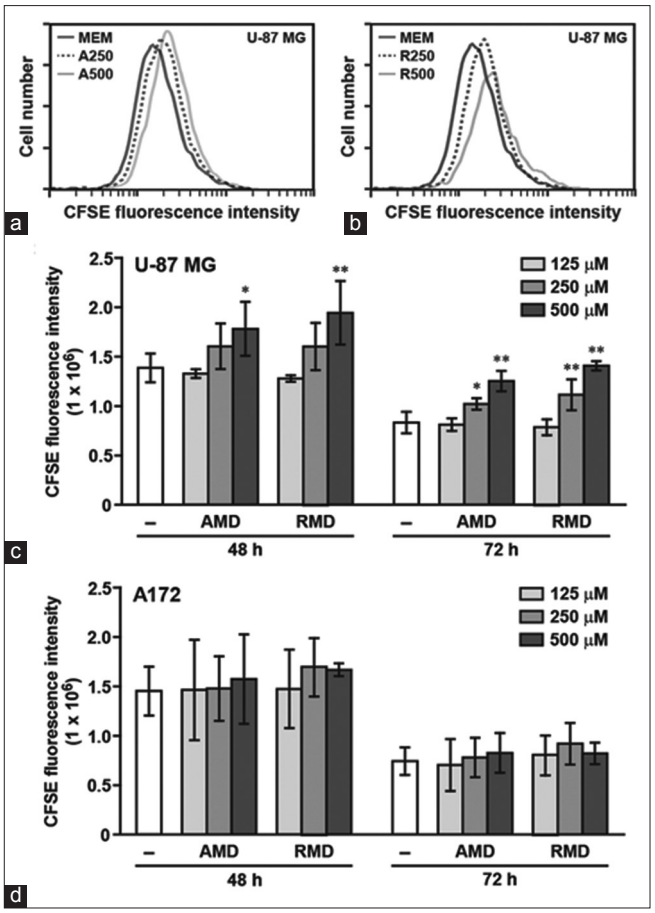

Figure 4: Inhibition of glioblastoma proliferation by amantadine and rimantadine. (a and b) Representative histograms showed higher CFSE fluorescence intensity in amantadine and rimantadine versus mock-treated cells in U-87 MG cells at 72 h. (c and d) Bar graphs represented the mean (± SD) fluorescence intensity of at least three independent experiments. Statistical analysis was performed using one-way ANOVA, followed by Dunnett's multiple comparisons test; *P < 0.05 and **P < 0.01 compared to the mock-treated group at each time point. AMD and A: Amantadine, CFSE: Carboxy-fluorescein diacetate succinimidyl ester; RMD and R: Rimantadine

study demonstrated apoptotic and antiproliferative effects of amantadine and rimantadine in two GBM cell lines. Both drugs improve symptoms of Parkinson's disease, indicating the penetration into the brain and the possibility to use as anti-cancers for GBM. The *in situ* rat brain perfusion technique indicates that rimantadine is much more readily to be transported across the blood-brain barrier than amantadine.[14] Concentrations of amantadine in postmortem brain tissues of Parkinson's patients with treatment duration longer than 10 days were ranged from 48.2 to 386 μM, while the concentrations in cerebrospinal fluid and serum were <17 μM, indicating the accumulation of the drug in brain tissues.[15] The authors also reported that the increased concentrations of amantadine in brain tissues are correlated with the treatment duration; therefore, it is possible to achieve the dose of amantadine and rimantadine with cytotoxic and antiproliferative effects.

The TC_{50} values of rimantadine on A172 cells were lower than the TC_{50} values of amantadine, indicating the higher toxicity of rimantadine. Interestingly, rimantadine showed

less cytotoxic in U-87 MG cells than A172 cells, while the antiproliferative effects were observed only in U-87 cells. Defect in apoptotic pathway contributes to malignancy. Induction of apoptosis is crucial to the suppression of tumorigenesis. Rimantadine increased the percentage of apoptotic cells in both GBM cell lines, while amantadine induced apoptosis only in U-87 MG cells, indicating the different sensitivity of GBM cell lines and superiority of rimantadine for GBM treatment. However, the toxic dose of rimantadine in the present study is higher than the toxic dose of amantadine in hepatocellular cancer cells at doses 10–75 μg/ml (about 50–400 μM).[12]

Genetic alterations such as tumor suppressor genes in cancers lead to unregulated cell proliferation. GBM has a rapid growth rate of 1.4 % per day and the equivalent volume doubling time was 49.6 days.[16] Both amantadine and rimantadine produced the rightward shift of CFSE fluorescence intensity compared to mock-treated cells, indicating the inhibition of cell division. Rimantadine at 500 μM inhibited U-87 MG cell proliferation at 48 and 72 h. However, amantadine suppressed cell proliferation at only 72 h, indicating the greater antiproliferative effect of rimantadine in U-87 MG cells. The antiproliferative concentrations of amantadine are similar to the effect on mouse T lymphocytes[17] which is much higher than the effect in hepatocellular cancer cells.[12] Amantadine at 300 μM displayed a reduction of Lyt-2+ (CD8+) T cell proliferation by 41% after 48 h.[17] Amantadine at 25–75 μg/ml (about 130–400 μM) inhibited human hepatocellular carcinoma cell growth following 48 and 72 h exposure by decreasing the expression of anti-apoptotic protein Bcl-2 and increasing the expression of pro-apoptotic protein Bax.[12] It is possible that rimantadine alters the expression of apoptotic-related proteins in GBM cell lines.

Cell cycle analysis revealed that U-87 MG cells treated with amantadine and rimantadine were largely accumulated in G0/G1 phase, which prevented cells from entering the next cycle. The number of cells in S and G2/M phases was decreased to compensate. Similarly, amantadine induced G0/G1 cell cycle arrest in hepatocellular carcinoma by downregulating cyclin E, cyclin D1, and CDK2 expression.[12] The arrest of cell cycle progression at the G0/G1 phase provides an opportunity for cells to undergo repair the damage.[18] If the damage cannot be repaired, the cell is eliminated through apoptosis. Therefore, cell cycle arrest at G0/G1 phase by both drugs results in the inhibition of cell cycle progression and the induction of apoptosis in GBM cells. Mice given a daily intraperitoneal injection of 30 and 60 mg/kg of amantadine for 3 days showed the increase of DNA damage in forebrain tissues.[9] Therefore, amantadine and rimantadine could downregulate the expression of cell cycle regulators and induce DNA damage in GBM, leading to G0/G1 arrest and apoptosis.

A172 and U-87 MG cells have different mutations on oncogenes and tumor suppressor genes (Manassas, VA, http://www.atcc.org), resulting in the difference in tumorigenicity and sensitivity to these drugs. U-87 MG cells are tumorigenic in nude mice, while A172 cells are not tumorigenic. Therefore, it is possible that U-87 MG cells could be more resistant to apoptotic effect than A172 cells. In the present study, cytotoxic and apoptotic effects of amantadine and rimantadine were more potent on A172 cells than U-87 MG cells. Both cells contain the same genomic mutation in tumor suppressor genes, cyclin-dependent kinase inhibitor 2A (CDKN2A) and PTEN. In addition, U-87 MG cells have a mutation in CDKN2C gene, a cell growth regulator that controls cell cycle G1 progression. In the present study, U-87 MG cells seemed more sensitive than A172 cells to antiproliferative effects of amantadine and rimantadine.

Rimantadine is better tolerated than amantadine in healthy volunteers and elderly patients and associated with a lower incidence of central nervous system adverse events.[7,8] The higher central nervous system adverse events in amantadine users were nervousness, lightheadedness, and difficulty concentrating on healthy subjects and confusion in elderly patients. Therefore, rimantadine may offer more promise than amantadine for GBM treatment.

Amantadine at low micromolar concentrations demonstrated the neuroprotective activity by inhibiting excitotoxicity in rat brain tissues.[19] This drug also increases glial cell line-derived neurotrophic factor expression in rat glioma cells and astroglia[20,21] and reduces the expression of pro-inflammatory factors from activated microglia.[21,22] Recent clinical studies demonstrate the neuroprotective effect of amantadine in executive dysfunction syndrome, dementia, and traumatic brain injury.[23-26] However, there has been no information about the neuroprotective effects of rimantadine. Therefore, amantadine may have less toxic effects on neurons than rimantadine if uses as an anticancer drug for GBM.

High doses or concentrations of amantadine and rimantadine were toxic to normal cells. Amantadine at doses 30 and 60 mg/kg for 3 days increased DNA damage in mice brain tissues.[9] Amantadine and rimantadine at 500 μM or more were toxic to MDCK kidney epithelial cells.[27] Amantadine at 300 μM reduced the proliferation of Lyt-2+ (CD8+) T cells by 41% after 48 h, while showed no effect on L3T4+ (CD4+) T cells.[17] Concentrations of amantadine and rimantadine used in the present study for apoptotic and antiproliferative effects were up to 500 μM and likely to affect normal brain cells. The investigation on the toxic effects of both drugs on primary neurons and astrocytes is warranted.

Both amantadine (1-adamantanamine) and rimantadine (α-methyl-1-adamantane-methylamine) are adamantine derivatives. The substitution of the amine group of amantadine with methenamine group in rimantadine reduced the median effective concentration against H3N2 influenza virus A from 2.0 μM to 0.36 μM.[27] 2-hydroxyrimantadine showed similar activity to amantadine, but the 3- and 4-hydroxyrimantadine showed only the modest inhibitory activity.[28] In contrast, 2-methyl-2-rimantadine exhibited a four-fold higher potency compared to rimantadine.[29] Interestingly, heterocyclic rimantadine analogs, pyrrolidine, and azetidine were about 10 folds more potent against influenza virus and 5- to 10-fold increase in cytotoxicity.[30] Therefore, these rimantadine analogs with high cytotoxic effects might be good candidate for anticancer effects. In contrast, the addition of piperidine structure on the adamantine moiety showed less or comparable antiviral activity to rimantadine, although the rigid carbon framework of piperidine fits into a lipophilic pocket in M2 proteins better than the free rotating structure.[27]

The modification of amantadine and rimantadine structure not only changes anti-influenza properties but also alters other properties. The methyl and ethyl substitution on the adamantine moiety increased dopamine transmission.[31] The

N-alkyl substitution of amantadine also increased the inhibition effect on nicotinic acetylcholine receptors.[32] Rimantadine is more potent than amantadine against *Trypanosoma brucei*. Aminoadamantane and aminoalkylcyclohexane derivatives increased trypanocidal activity.[33,34] However, these studies did not report the cytotoxicity of these compounds.

CONCLUSION

Rimantadine showed greater apoptotic than amantadine on both A172 and U-87 MG GBM cell lines, while both drugs demonstrated antiproliferative effects on U-87 MG cells. GBM is quite resistant to standard chemotherapy. The structure of these drugs is quite similar to memantine, an anti-Alzheimer's drug. Further structure-activity relationship studies could lead to the development of new anticancer drugs for GBM.

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