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Bcl-2 siRNA Augments Taxol Mediated Apoptosis in Human Glioblastoma Cell Lines

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Abstract

The small interfering RNA (siRNA) is a useful tool to knockdown the mRNA and thus protein level of a target gene. Taxol is a potent anti-neoplastic drug that strongly binds to β-tubulin to prevent tumor cell division and promote cell death. The anti-apoptotic molecule Bcl-2 is upregulated in glioblastomas to protect from apoptosis. The aim of our present study was to downregulate Bcl-2 during low dose of taxol treatment to induce apoptosis very effectively. Using Bcl-2 siRNA, we knocked down the cognate mRNA and subsequently protein levels in two human glioblastoma cell lines. The cells in culture were treated with either taxol (100 nM) or Bcl-2 siRNA (100 nM) or both for 72 h. FACS analysis and TUNEL assay demonstrated apoptosis in 40-50% of cells treated with taxol and Bcl-2 siRNA together. Immunofluorescence for calpain and caspase-3 depicted increased expression and co-localization of both molecules. Fluorometric assays showed increased levels of intracellular free Ca^{2+}, calpain, and caspase-3 to promote apoptosis. Western blots demonstrated dramatic increases in the levels of TRADD, FADD, Bid, Bax, active caspases, DFF40, cleaved fragments of lamin, fodrin and PARP during apoptosis. The events related to apoptosis were prominent more in combination therapy than in either treatment alone. Our study demonstrated that combination of taxol and Bcl-2 siRNA was highly effective for inducing apoptosis in glioblastoma cell lines through induction of caspase mediated signaling pathways. Treatment with combination of taxol and Bcl-2 siRNA offers a novel therapeutic tool for controlling growth of glioblastomas.

Introduction

Apoptosis plays important roles in the development and maintenance of homeostasis and in the maturation of nervous and immune systems. Apoptosis is regulated by a series of complex biochemical events that are controlled by an evolutionarily conserved program. Dysregulation of apoptotic mechanisms plays an important role in the pathogenesis and progression of various cancers as well as in the responses of tumors to therapeutic interventions. Cancer cells are protected from apoptosis by upregulation of various anti-apoptotic molecules, such as B-cell lymphoma-2 (Bcl-2).

Taxol is a novel chemotherapeutic drug widely used in various forms of cancers including glioblastoma. Taxol strongly binds to the β-subunit of tubulin, which is the building block of microtubules. The dynamic instability of microtubules affects the positioning of chromosomes during replication and finally inhibits cell division. Taxol also induces apoptosis in a wide spectrum of cancer cells by caspase-dependent and -independent apoptosis mechanisms. Taxol chemotherapy may be an effective treatment for aggressive brain tumors because it inhibits cell division by preventing microtubluar restructuring and induces apoptosis. However, taxol also inhibits normal cell division and causes undesirable side effects for patients. A low dose of taxol treatment, which inhibits only the fast dividing tumor cells with less toxicity to healthy normal cells, would be highly desirable.

RNA interference (RNAi) through small interfering RNA (siRNA) is an incredibly powerful tool to knockdown a gene's message, and subsequently the protein level of the targeted gene. The siRNA mediated gene knockdown is a process of highly sequence-specific, post-transcriptional gene silencing initiated by a synthetic double stranded RNA (dsRNA) molecule. Silencing of several unwanted genes would be a promising step to regulate uncontrolled cell division and induce apoptosis in tumor cells. Bcl-2 is an anti-apoptotic molecule and is upregulated in highly invasive brain tumor cells in order to protect the tumor cells from apoptosis. Bcl-2 exerts a survival function in response to a wide range of apoptotic stimuli through inhibition of mitochondrial cytochrome c release. Furthermore, Bcl-2 protects neuronal cells against taxol-induced apoptosis by inducing multi-nucleation. Introduction of the Bcl-2 siRNA into cells triggers degradation of Bcl-2 endogenous mRNA and thus downregulates the functional protein level. The aim of our present investigation was to knockdown the Bcl-2 level using gene specific siRNA in human glioblastoma cell lines in order to induce apoptosis in a more efficient and effective manner during low dose taxol treatment. We employed two highly invasive human glioblastoma cell lines U-138MG and U-251MG for our experiments.

Figure 1

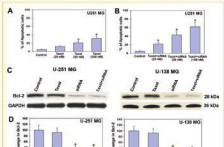

Figure 1 (A) TUNEL assay for apoptotic cells after treating U-251MG cells with 25 nM, 50 nM, and 100 nM taxol. (*p < 0.001, N=3). **(B)** TUNEL assay for apoptotic cells after treating U-251MG cells with combination each 25 nM, 50 nM and 100 nM taxol and BCl-2 siRNA. (*p < 0.001, N=3). **(C)** Western blot analysis for the expression of Bcl-2 in the cell preparation of U-138MG and U-251MG glioblastoma cells after treatment with 100 nM taxol or 100 nM Bcl-2 siRNA (human specific) or both. The membranes were reprobed for GAPDH content to demonstrate that similar amounts of protein were loaded in each lane. **(D)** Quantitative evaluation of the percentage expression of Bcl-2 protein in U-138MG and U-251MG cells when compared with non-treated control cells. The Western blots were quantified using Image-J software to assess the percentage knockdown of Bcl-2 protein after treatment with taxol or Bcl-2 siRNA or both (*p < 0.001, N=6). Western blot analysis demonstrated about 60% knockdown of functional Bcl-2 protein level with cognate siRNA.

Figure 2

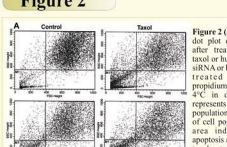

Figure 2 (A). Flow cytometry dot plot of U-251MG cells after treatment with either taxol or human specific Bcl-2 siRNA or both. The cells were treated with 50 g/ml propidium iodide for 30 min at 4°C in dark. Column M1 represents the apoptotic cell population. A marked increase of cell population in the M1 area indicates increased apoptosis after treatment with taxol or transfection with Bcl-2 siRNA or both. The combined treatment of taxol and Bcl-2 siRNA resulted in more apoptotic cells than treatment with either alone.

(B). FACS histogram of U-251MG cells after treatment with taxol or transfection with Bcl-2 siRNA. The prominent increase of sub-G1 phase population of cells (M1) indicates increased apoptosis after treatment with taxol or Bcl-2 siRNA or both.

(C). Quantitative representation of FACS data for five and apoptotic cells. Data are representative of 4 independent experiments (*p<0.001 when compared to the control mean values and #p<0.001 when compared to taxol or Bcl-2 siRNA mean values).

Figure 3

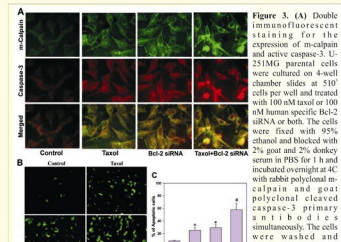

Figure 3. (A) Double immunofluorescent staining for the expression of m-calpain and active caspase-3. U-251MG parental cells were cultured on 4-well chamber slides at 5×10^3 cells per well and treated with 100 nM taxol or 100 nM human specific Bcl-2 siRNA or both. The cells were fixed with 95% ethanol and blocked with 2% goat and 2% donkey serum in PBS for 1 h and incubated overnight at 4C with rabbit polyclonal m-calpain and goat polyclonal cleaved caspase-3 primary antibodies simultaneously. The cells were washed and incubated with FITC conjugated anti-rabbit and Texas red conjugated anti-goat secondary antibodies at room temperature for 1 h. A marked increase was noticed in the expression of both m-calpain and active caspase-3 after treatment with taxol or Bcl-2 siRNA or both. The staining of both molecules was more prominent after combined treatment of taxol and Bcl-2 siRNA. Merged pictures demonstrated the simultaneous expression of m-calpain and caspase-3 as well as several apoptotic cells. **(B)** Fluorescent TUNEL assay for apoptotic cells after treating U-251MG cells with taxol or transfection with Bcl-2 siRNA or both. The results demonstrated a significant increase of apoptosis in U-251MG cells after treatment with taxol or Bcl-2 siRNA. The combined treatment with both molecules resulted in more prominent apoptotic cell death **(C)** Quantitative evaluation of TUNEL assays using Image Pro-Discovery software. Data are representative of 4 independent experiments in duplicate (*p<0.001 when compared to the control mean values and #p<0.001 when compared to taxol or Bcl-2 siRNA mean values).

Figure 4

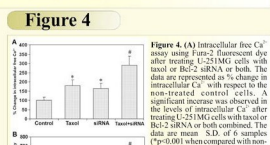

Figure 4. (A) Intracellular free Ca^{2+} assay using Fura-2 fluorescent dye after treating U-251MG cells with taxol or Bcl-2 siRNA or both. The data are represented as % change in intracellular Ca^{+} with respect to the non-treated control cells. A significant increase was observed in the levels of intracellular Ca^{2} after treating U-251MG cells with taxol or Bcl-2 siRNA or both combined. The data are mean S.D. of 6 samples (*p<0.001 when compared with non-treated controls and #p<0.001 when compared to taxol or Bcl-2 siRNA mean values).

(B) Fluorometric protease activity assay for total calpain after treating U-251MG cells with 100 nM taxol or 100 nM human specific Bcl-2 siRNA or both. The data are represented as % change in total calpain activity with respect to the non-treated control cells. A significant increase was observed in the activity of calpain after treating U-251MG cells with taxol or Bcl-2 siRNA or both. The data are mean S.D. of 6 samples (*p<0.001 when compared with non-treated controls and #p<0.001 when compared to taxol or Bcl-2 siRNA mean values).

(C) Fluorometric protease activity assay for Caspase-3 after treating U-251MG cells with taxol or Bcl-2 siRNA or both. The data are represented as % change in caspase 3 activities with respect to the non-treated control cells. A significant increase was observed in the activity of caspase-3 after treating U-251MG cells with taxol or Bcl-2 siRNA or both. The data are mean S.D. of 6 samples (*p<0.001 when compared with non-treated controls and #p<0.001 when compared to taxol or Bcl-2 siRNA mean values).

Figure 5

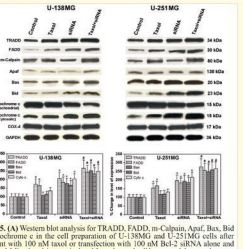

Figure 5. (A) Western blot analysis for TRADD, FADD, m-Calpain, Apaf, Bax, Bid and cytochrome c in the cell preparation of U-138MG and U-251MG cells after treatment with 100 nM taxol or transfection with 100 nM Bcl-2 siRNA alone and combined. Mitochondria were isolated from the total cell lysate, and the cytochrome c was determined in both mitochondrial and cytosolic fraction. The nitrocellulose membranes were reprobed and analyzed for GAPDH and COX-4 to demonstrate that similar amounts of protein were loaded in each lane. There were significant increases in TRADD, FADD, m-Calpain, Bax, Bid and cytosolic fraction of cytochrome c after treating both cells with taxol or Bcl-2 siRNA or both, indicating triggering of apoptotic signaling molecules and an increased rate of cytochrome c release. Data are representative of 3 independent experiments. **(B)** Quantitative evaluation of the percentage expression of TRADD, FADD, Bax, Bid and Cytochrome c (cytosolic) in U-138MG and U-251MG cells after treatment with taxol or Bcl-2 siRNA alone or both agents combined. The Western blots were quantified using Image-J software. Data are representative of 3 independent experiments. (*p<0.001 when compared with non-treated controls and #p<0.001 when compared with taxol or Bcl-2 siRNA mean values).

Figure 6

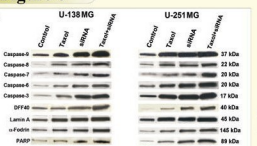

Figure 6. (A) Western blot analysis for active caspase-9, -8, -7, -6 &-3, DFF40, cleaved lamin A, cleaved α-fodrin and active PARP in the cell lysate of U-138MG and U-251MG cells after treatment with 100 nM taxol or transfection with 100 nM Bcl-2 siRNA alone, and both combined. PARP was determined in the nuclear fraction. The nitrocellulose membranes were reprobed and analyzed for GAPDH content to demonstrate that similar amounts of protein had been loaded in each lane. Significant increases were found in the levels of active caspases, DFF40, cleaved lamin A, cleaved α-fodrin and PARP after treating U-138MG and U-251MG cells with taxol or Bcl-2 siRNA or both, indicating increased rate of caspase mediated apoptosis. Data are representative of 3 independent experiments. **(B)** Quantitative evaluation of the percentage expression of active caspase 3, DFF40, Lamin A, α-Fodrin and PARP in U-138MG and U-251MG cells after treatment with taxol or Bcl-2 siRNA alone or both agents combined. The Western blots were quantified using Image-J software. Data are representative of 3 independent experiments. (*p<0.001 when compared with non-treated controls and #p<0.001 when compared with taxol or Bcl-2 siRNA mean values).

Figure 7

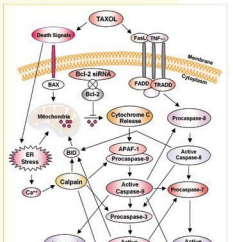

Figure 7. Mechanistic aspects of taxol and Bcl-2 siRNA on the induction of calpain and caspase mediated apoptotic pathway. The induction of apoptosis occurs through modification of death receptor/death ligand activity of the cell surface molecules, resulting in the activation of calpain and caspase mediated apoptotic pathway. Bcl-2 siRNA downregulates the anti-apoptotic molecule Bcl-2 and accelerates the release of cytochrome c from mitochondria. The association of cytosolic cytochrome c with procaspase-9 and APAF-1 processes procaspase-9 to its active form, which then initiates the intrinsic pathway of caspase-mediated apoptosis. Procaspase-3 is cleaved to its active form by elevated calpain, caspase-9 as well as caspase-8. The active caspase-3 in turn processes α-Fodrin, DFF45 and PARP which cause nuclear fragmentation, cytoplasmic membrane blebbing, and DNA fragmentation leading to apoptosis.

Conclusions

- *Transfection of U-138 and U-251 glioblastoma cells with Bcl-2 siRNA resulted knockdown of Bcl-2 functional protein levels.*
- *Double immunofluorescence for m-calpain and active caspase-3 demonstrated increased staining and co-localization of both molecules as well as apoptosis after knockdown of Bcl-2.*
- *Western blots demonstrated increases in active caspases, DFF40, cleaved fragments of lamin, fodrin and PARP during apoptosis.*
- *Combination treatment of Taxol and Bcl-2 siRNA is more effective to induce apoptosis than either agents alone.*
- *Combination of taxol and Bcl-2 siRNA offers a novel therapeutic tool for controlling growth of glioblastomas.*

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