Original article

Sodium valproate induces mitochondria-dependent apoptosis in human hepatoblastoma cells

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Background Sodium valproate inhibits proliferation in neuroblastoma and glioma cells, and inhibits proliferation and induces apoptosis in hepatoblastoma cells. Information describing the molecular pathways of the antitumor effects of sodium valproate is limited; therefore, we explored the mechanisms of action of sodium valproate in the human hepatoblastoma cell line, HepG2.

Methods The effects of sodium valproate on the proliferation of HepG2 cells were evaluated by the Walsh-schema transform and colony formation assays. Sodium valproate-induced apoptosis in HepG2 cells was investigated with fluorescence microscopy to detect morphological changes; by flow cytometry to calculate DNA ploidy and apoptotic cell percentages; with Western blotting analyses to determine c-Jun N-terminal kinases (JNK), p-JNK, Bcl-2, Bax, and caspase-3 and -9 protein expression levels; and using JC-1 fluorescence microscopy to detect the membrane potential of mitochondria. Statistical analyses were performed using one-way analysis of variance by SPSS 13.0 software.

Results Our results indicated that sodium valproate treatment inhibited the proliferation of HepG2 cells in a dose-dependent manner. Sodium valproate induced apoptosis in HepG2 cells as it: caused morphologic changes associated with apoptosis, including condensed and fragmented chromatin; increased the percentage of hypodiploid cells in a dose-dependent manner; increased the percentage of annexin V-positive/propidium iodide-negative cells from 9.52% to 74.87%; decreased JNK and increased phosphate-JNK protein expression levels; reduced the membrane potential of mitochondria; decreased the ratio of Bcl-2/Bax; and activated caspases-3 and -9.

Conclusion Sodium valproate inhibited the proliferation of HepG2 cells, triggered mitochondria-dependent HepG2 cell apoptosis and activated JNK.

Liver cancer is the third most common cancer in the world and a leading cause of cancer morbidity and mortality. Hepatocellular carcinoma (HCC) is a primary malignant tumor of the liver that is associated with poor prognosis. Traditional treatment regimes for HCC include surgical techniques and various chemotherapies; however, significant survival benefit from these protocols is unlikely. In particular, chemotherapy has variable effects as HCC is extremely chemoresistant; therefore, the search for new reliable chemotherapy regimens must be actively pursued. Recently, sodium valproate was shown to inhibit the proliferation of cancer cells in neuroblastoma and glioma, and effect the proliferation and apoptosis of human hepatoblastoma HepG2 cells. Sodium valproate is a histone deacetylase inhibitor that has been used to treat epilepsy. Valproic acid possesses anti-manic properties and is an important mood stabilizer in the treatment of bipolar disorder. It inactivates the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) through the inhibition of GABA transaminase and has acute inhibitory effects on neuronal excitation in the central nervous system. Presently, information describing the molecular mechanisms of the antitumor effects of sodium valproate is limited.

Mitogen-activated protein kinases (MAPKs) are a family of proline-directed Ser/Thr kinases activated by dual phosphorylation. Three MAPK groups have been the focus of intense study: the extracellular signal-regulated kinases (ERKs), the c-Jun N-terminal kinases (JNKs), and the p38 MAPKs. The ERK cascade is activated by growth factors and is critical for proliferation and survival. The p38 and JNK pathways are simultaneously activated in response to a variety of cellular and environmental stresses such as pro-inflammatory cytokines, genotoxic agents, and apoptosis.
In the present study, we demonstrated that sodium valproate inhibited the proliferation of human hepatoblastoma HepG2 cells and induced apoptosis by reducing the membrane potential of mitochondria and activating caspases-3/9. Furthermore, we found that JNK was involved in the antitumor activity of sodium valproate.

**METHODS**

**Cells and reagents**
HepG2 cells (human hepatoblastoma cell line) were maintained in RPMI 1640 (Gibco Corporation, Carlsbad, California, USA) supplemented with 10% newborn bovine serum (Gibco Corporation) at 37ºC and 5% CO2. Sodium valproate was dissolved in PBS to make a stock solution of 30 mmol/L. The primary antibodies used for Western blotting analyses were anti-caspase-3 (9662; Cell Signaling), anti-caspase-9 (9502; Cell Signaling), anti-JNK (9525; Cell Signaling), anti-P-JNK (9251; Cell Signaling), anti-Bcl-2 (2876; Cell Signaling), anti-Bax (2772; Cell Signaling), anti-GAPDH (1:1000 dilution in blocking buffer) overnight at 4ºC. Subsequently, membranes were washed in TBST and reacted with appropriate antibodies for JNK, phospho-JNK, Bcl-2, Bax, caspase-9, caspase-3, or GAPDH (1:1000 dilution in blocking buffer) overnight at 4ºC. Proteins were visualized by incubation with SuperSignal West Pico reagent (NCI5079 Thermo) and exposure to radiograph film (Kodak, Rochester, USA).

**Western blotting**
HepG2 cells were treated with 0, 2, 4, 6, and 8 mmol/L sodium valproate for 48 hours. Whole cell lysates were prepared by adding 2 × SDS sample buffer. Equal amounts of protein were electrophoresed on 10% SDS-PAGE gels and transferred to PVDF membranes. Membranes were blocked for 60 minutes at room temperature with 5% nonfat dry milk/TBS-Tween 20 (TBST) and reacted with appropriate antibodies for JNK, phospho-JNK, Bcl-2, Bax, caspase-9, caspase-3, or GAPDH (1:1000 dilution in blocking buffer) overnight at 4ºC. Proteins were visualized by incubation with SuperSignal West Pico reagent (NCI5079 Thermo) and exposure to radiograph film (Kodak, Rochester, USA).

**Flow cytometry**
Flow cytometry (Beckman Coulter, Fullerton, California, USA) was used to identify apoptotic cells by DNA fragmentation analysis, and to determine the apoptotic rate. For DNA fragmentation analysis, HepG2 cells were incubated with 0, 2, 4, 6, and 8 mmol/L sodium valproate for 48 hours, harvested, washed with PBS, and fixed in 70% ice-cold ethanol overnight. Fixed cells were incubated with 20 U/ml RNase I and 50 μg/ml propidium iodide (PI) for 30 minutes. Cells in the sub-G1 phase of the cell-cycle were identified as apoptotic. To determine the apoptotic rate, HepG2 cells were seeded in 6-well plates at a density of 2 × 104 cells/ml, incubated with sodium valproate for 48 hours, and collected. Annexin V-FITC/PI (Becton Dickinson, USA) staining was performed following the manufacturer’s protocol. Phosphatidyl serine translocation to the cell surface is an indicator of early apoptotic cells; therefore, annexin V-positive, PI-negative cells were identified as apoptotic. The apoptotic rate was determined using CellQuest software (FCM, Becton Dickinson).

**Fluorescence microscopy**
Sodium valproate-induced apoptosis in HepG2 cells was visualized using Hoechst 33258 staining. Cells were treated with 5 mmol/L sodium valproate for 48 hours, harvested, and smeared on slides. The slides were air dried, fixed in methanol/acetone (3:1, v/v), and stained with Hoechst 33258 (5 µg/ml) for 20 minutes. Nuclear morphological changes were examined using fluorescence microscopy (DFC480; Leica Microsystems, Wetzlar, Germany).

**Analysis of mitochondrial membrane potential**
HepG2 cells were treated with 0, 2, and 4 mmol/L sodium valproate for 48 hours, harvested, washed with PBS, fixed in JC-1 solution for 30 minutes at 37ºC, and smeared on slides. The membrane potential of mitochondria was examined under a fluorescence microscope.

**Statistical analysis**
All assays were performed in triplicate. Data are expressed as mean ± standard deviation (SD). Statistical analyses were performed using one-way analysis of variance (ANOVA) by SPSS 13.0 software (SPSS, USA). A value of P <0.05 was considered statistically significant.

**RESULTS**
Sodium valproate inhibits HepG2 cell proliferation
The proliferation of HepG2 cells was significantly...
inhibited in a dose-dependent manner by exposure to sodium valproate for 48 hours. The IC$_{50}$ value of sodium valproate for HepG2 cells was 5.38 mmol/L. In addition, colony formation was significantly decreased when HepG2 cells were exposed to various concentrations of sodium valproate for 7 days (Figure 1).

**Sodium valproate induces apoptosis in HepG2 cells**

Inhibition of cell proliferation could result from the induction of apoptosis, cell growth arrest, and/or the inhibition of growth. We investigated whether sodium valproate induced apoptosis in HepG2 cells. The percentage of cells in the hypodiploid DNA peak (sub-G1 population) and annexin V-FITC/PI double staining were used as indicators of apoptosis. Sodium valproate treatment increased the sub-G1 cell population and the percentage of annexin V-positive/PI-negative cells (from 9.52% to 74.87%) (Figure 2).

**Figure 1.** Sodium valproate-induced inhibition of HepG2 cell colony formation. Data represent one of three experiments yielding similar results.

**Figure 2.** Induction of apoptosis in HepG2 cells by sodium valproate. A: Propidium iodide (PI) staining shows the cellular DNA content in sodium valproate exposed cells. HepG2 cells were treated with 0, 2, 4, 6, and 8 mmol/L sodium valproate for 48 hours. Data shown are representative of 3 different experiments. B: Sodium valproate-induced apoptosis was determined by annexin V-FITC and PI staining. HepG2 cells were treated with 0, 2, 4, 6, and 8 mmol/L sodium valproate for 48 hours. Data represent one of three experiments yielding similar results.
In addition, cells were stained with Hoechst 33258 to detect the morphologic changes of apoptotic nuclei using fluorescence microscopy. Following exposure to 5 mmol/L sodium valproate (approx. IC50) for 48 hours, HepG2 cells exhibited typical apoptotic characteristics including cell shrinkage, apoptosis-chromatin condensation, and apoptotic body formation.

**Effect of sodium valproate on intracellular signaling protein expression levels**

To explore the molecular mechanisms of sodium valproate-induced apoptosis, HepG2 cells were treated with different concentrations of sodium valproate for 48 hours, and JNK and p-JNK protein expression levels were detected. Western blotting indicated that sodium valproate increased the phosphorylation of JNK and decreased JNK expression in a dose-dependent manner (Figure 3A). As the intrinsic pathway of apoptosis is regulated by members of the Bcl-2 protein family, Bcl-2 and Bax protein expression levels were also analyzed. Western blotting revealed that sodium valproate caused a significant decrease in Bcl-2 expression, and that Bax was unaffected. The ratio of Bcl-2/Bax was reduced (Figure 3B).

**Figure 3.** Effects of sodium valproate on intracellular signaling protein expression levels in HepG2 cells. A: Sodium valproate activates the JNK pathway in HepG2 cells. HepG2 cells were treated with 0, 2, 4, 6, and 8 mmol/L sodium valproate for 48 hours. The effects of sodium valproate on JNK protein expression levels were evaluated by Western blotting. B: Sodium valproate caused a significant decrease in Bcl-2 but not Bax protein levels. HepG2 cells were treated with 0, 2, 4, 6, and 8 mmol/L sodium valproate for 48 hours. Bcl-2 and Bax protein expression levels were detected by Western blotting. Data represent one of three experiments yielding similar results.

**Sodium valproate activates caspases-3 and -9 and changes the membrane potential of mitochondria in HepG2 cells**

To investigate the role of caspases in sodium valproate-induced apoptosis, we investigated the activation of caspases-3 and -9 in HepG2 cells treated with different concentrations of sodium valproate for 48 hours. The results showed that sodium valproate activated caspases-3 and -9 in a dose-dependent manner (Figure 4A).

In addition, cytofluorometry was used to analyze the membrane potential of the mitochondria in HepG2 cells after sodium valproate treatment using the lipophilic cationic probe, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolocarbocyanine iodide (JC-1).16 This membrane-permeable fluorochrome can penetrate cells and its fluorescence is a reflection of the mitochondrial membrane potential. When live cells are incubated with JC-1, mitochondrial membrane polarization allows JC-1 to selectively enter mitochondria which causes the reversible formation of JC-1 aggregates. JC-1 aggregates showed a spectral shift in emitted light from 530 nm (emission of JC-1 monomeric form; green) to 590 nm (emission of J-aggregate; green-orange) upon excitation at 490 nm. Our data showed reduced levels of orange fluorescence in HepG2 cells treated with 2 and 4 mmol/L sodium valproate for 48 hours compared with the control (Figure 4B).

**DISCUSSION**

In this study, we investigated the effect of sodium valproate on the human hepatoblastoma HepG2 cell line. We found that sodium valproate effectively inhibited the proliferation of HepG2 cells and induced apoptosis in a dose-dependent manner. Our results suggest that sodium valproate has potential as an antitumor drug.

Previous studies indicate that sodium valproate induces cell death.17 In accordance with these reports, our results showed that sodium valproate induced apoptosis and caused typical apoptotic morphology including cell shrinkage, nuclear condensation, and nuclear fragmentation in HepG2 cells. Taken together, these data indicate that the antitumor effect of sodium valproate on HepG2 cells is due to the induction of cell apoptosis.

We investigated the mechanism of action of sodium valproate on HepG2 cells. Sodium valproate phosphorylated and activated JNK, inhibited Bcl-2 protein expression, and had no effect on Bax. Bcl-2 and Bax are members of the Bcl-2 family of proteins which are central regulators of the mitochondrial pathway of apoptosis. The Bcl-2 family is composed of pro- and antiapoptotic proteins. Antiapoptotic members such as Bcl-2 promote cell survival by inhibiting the functions of the proapoptotic proteins. Under normal conditions, Bax is localized to the cytosol; however, in response to death stimuli, Bax undergoes a conformational change that triggers its translocation to and insertion into the outer mitochondrial membrane. This leads to the permeabilization of the outer mitochondrial membrane and the
release of proapoptotic proteins, including cytochrome c. Released cytochrome c interacts with apoptotic protease activating factor-1 and pro-caspase-9 to form the apoptosome. This generates mature caspase-9 and begins a proteolytic cascade, ultimately resulting in cell death. We observed a sodium valproate-induced decrease in the ratio of Bcl-2/Bax in HepG2 cells and propose that this resulted in the initiation of the apoptotic pathway.

Mitochondria play a crucial role in the apoptotic signal transduction pathway. The change in membrane potential of mitochondria activates both caspases-3 and -9 and is an important upstream event of mitochondrial-dependent apoptosis. In our study, sodium valproate activated caspases-3 and -9 in HepG2 cells in a dose-dependent manner. This suggests that sodium valproate-induced apoptosis in hepatoblastoma cells results from caspase activation and the mitochondrial apoptotic pathway.

In summary, we found that sodium valproate inhibits the proliferation and induces apoptosis of HepG2 cells through an intracellular signaling pathway involving the activation of JNK, the Bcl-2 proteins, and caspases-3 and -9. Further studies are needed to fully understand the mechanism of action of sodium valproate and should investigate the expression of other Bcl-2 anti-apoptosis family members such as Bcl-xl, Mcl-1, and the BH-3 domain-only proteins; the translocation of Bax from the cytosol to mitochondria; and the relationship between JNK activation, Bcl-2 proteins, and the mitochondria-dependent cell apoptosis signaling pathway. Our data identify sodium valproate as a potential novel therapeutic agent for use against human hepatoblastoma cells. Furthermore, as sodium valproate is a drug traditionally used to treat epilepsy, our study may provide useful information describing the relationship between nervous disorders and cancer.

REFERENCES


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