Devazepide, a nonpeptide antagonist of CCK receptors, induces apoptosis and inhibits Ewing tumor growth

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The Ewing family of tumors is a group of highly malignant tumors that mainly arise in bone and most often affect children and young adults in the first two decades of life. Despite the use of multimodal therapy, the long-term disease-free survival rate of patients with Ewing tumors is still disappointingly low, making the discovery of innovative therapeutic strategies all the more necessary.
We have recently shown that cholecystokinin (CCK), a neuroendocrine peptide, involved in many biological functions, including cell growth and proliferation, is a relevant target of the EWS/FLI1 oncoprotein characteristic of Ewing tumors. CCK silencing inhibits cell proliferation and tumor growth *in vivo*, suggesting that CCK acts as an autocrine growth factor for Ewing cells. Here, we analyzed the impact of two CCK receptor antagonists, devazepide (a CCK1-R antagonist) and L365 260 (a CCK2-R antagonist), on the growth of Ewing tumor cells. Devazepide (10 μmol/l) inhibited cell growth of four different Ewing tumor cells *in vitro* (range 85–88%), whereas the effect of the CCK2-R antagonist on cell growth was negligible. In a mouse tumor xenograft model, devazepide reduced tumor growth by 40%. Flow cytometry experiments showed that devazepide, but not L365 260, induced apoptosis of Ewing tumor cells. In summary, devazepide induces cell death of Ewing tumor cells, suggesting that it could represent a new therapeutic approach in the management of Ewing's tumor patients. *Anti-Cancer Drugs* 20:527–533 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Tumors of the Ewing family mainly affect children and young adults during the first or second decade of life. In these patients, the long-term disease-free survival rate is disappointingly low, even when combined therapies are used, such as chemotherapy, radiotherapy, surgery, and bone marrow transplantation (reviewed in Ref. [1]). Thus, it is clearly necessary to look for new effective strategies and drugs to improve the prognosis for Ewing tumors and to reduce the side effects originated by the therapies currently employed.

In the Ewing family of tumors, balanced chromosomal translocations generate chimeric transcription factors that are implicated in the pathogenesis of these tumors [2,3]. Although important advances have been made in recent years, in identifying genes regulated by the specific fusion proteins and defining the molecular biology behind the formation of these tumors [4], few new molecular targets or therapies have been generated.

We recently showed that the neuroendocrine peptide cholecystokinin (CCK) is a target of the EWS/FLI1 oncoprotein and, as a result, it is expressed strongly in Ewing cells and tumors. In an inducible model in which the expression of a CCK-specific small interference RNA produces the knockdown of CCK in Ewing cells; cell proliferation and tumor growth is impaired *in vivo*, suggesting that CCK acts as an autocrine growth factor in Ewing cells [5].

CCK is primarily synthesized as a preprohormone, and it undergoes a series of enzymatic reactions to produce the fully active C-terminal amidated and tyrosine-sulfated octapeptide termed CCK8 [6,7]. The biological function of CCK is mediated by its binding to two specific receptors, CCK1R and CCK2R, which belong to the seven transmembrane receptor superfamily [8]. These receptors have been classified according to their pharmacological profile for agonists (CCK and gastrin) and for synthetic competitive antagonists. As a result, CCK1R is highly specific for sulfated CCK8 and for the nonpeptide antagonist devazepide (L364 718), whereas the CCK2R binds to CCK8 and gastrin with similar affinity and it is specifically antagonized by the nonpeptide L365 260 [8]. Binding of mature CCK8 to the receptors

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activates receptor-coupled G-proteins, which leads to an increase in the levels of cAMP and the activation of protein kinase A. Ultimately, this interaction results in the activation of the jun-fos transcription factor and early growth-promoting genes [9–11].

Here, we have analyzed the impact of the CCK receptor antagonists devazepide and L365 260 on the growth of Ewing tumor cells. We show that devazepide, but not L365 260, inhibits growth of Ewing tumor cells both *in vitro* and *in vivo* by inducing apoptosis. Our results open the possibility that devazepide could be used as a new therapeutic approach in the treatment of Ewing tumors.

Materials and methods

Cell lines and tumors

The Ewing tumor cell lines A673 (# CRL-1598), SK-N-MC (# HTB-10), and SK-PN-DW (# CRL-2139) were all purchased from the ATCC (Manassas, Virginia, USA). Ewing tumor cell lines TC-71 were a generous gift from Dr S. Navarro (University of Valencia).

Cell growth assays

Cells were plated at a density of 25 000 cells per well in six-well plates (BD Biosciences, San Jose, California, USA) and allowed to attach for 24–48 h. Where indicated, cells were exposed to devazepide, L365 260 or vehicle alone (dimethyl sulfoxide, DMSO) for different periods of time. Cell growth was determined by crystal violet staining of viable cells [12]. Devazepide (L364 718) and L365 260 were kindly provided by ML Laboratories Plc (Liverpool, UK).

Analysis of cell cycle and apoptosis

A673 and SK-N-MC Ewing cells were plated at a density of 150 000 or 300 000 cells in 100 mm dishes (BD Biosciences) and allowed to attach for 24–48 h. Cells were then incubated with the different drugs or vehicle for 24–96 h. Subsequently, both floating and adherent cells were collected and analyzed for DNA content and apoptosis. For analysis of DNA content, cells were fixed with 70% cold ethanol, incubated with RNase (100 μg/ml) and propidium iodide (40 μg/ml) for 30 min at 37°C, and analyzed by flow cytometry (FACSCAN, BD Biosciences). To detect and quantify apoptosis, cells were labeled with annexin V-FITC (Annexin-V-Fluos Staining kit, Roche Applied Science, Basel, Switzerland), according to the manufacturer's instructions, and then they were assayed by flow cytometry. The results were analyzed with WinMDI Version 2.8 software (The Scripps Research Institute, La Jolla, California, USA).

Tumor formation assay in nude mice

Athymic 6-week-old female BALB/c *nu/nu* mice (Harlan Ibérica, Barcelona, Spain) were used in these experiments, which were all carried out in accordance with Institutional and European Union guidelines. A673 cells were washed twice in PBS and resuspended at a density of 5×10^7 cells/ml before they were subcutaneously injected into the left flanks of the mice (0.1 ml). The animals were then kept under pathogen free conditions and observed daily for any visible signs of tumors at the injection sites. Devazepide was dissolved in DMSO at 2.5 mg/ml and diluted 1/10 in 0.5% methylcellulose in serum saline and administered daily by subcutaneous injection (0.1 ml) into the right flank of the animal (final dose: 10 mg/kg/day). Control animals received the vehicle alone. The tumor volume was measured every 2 or 3 days and calculated using the formula $L \times W \times \pi/6$, where L is the length and W is the width of the tumors. Mice that did not develop a subcutaneous mass were excluded from the calculation of the tumor diameter.

Statistical analysis

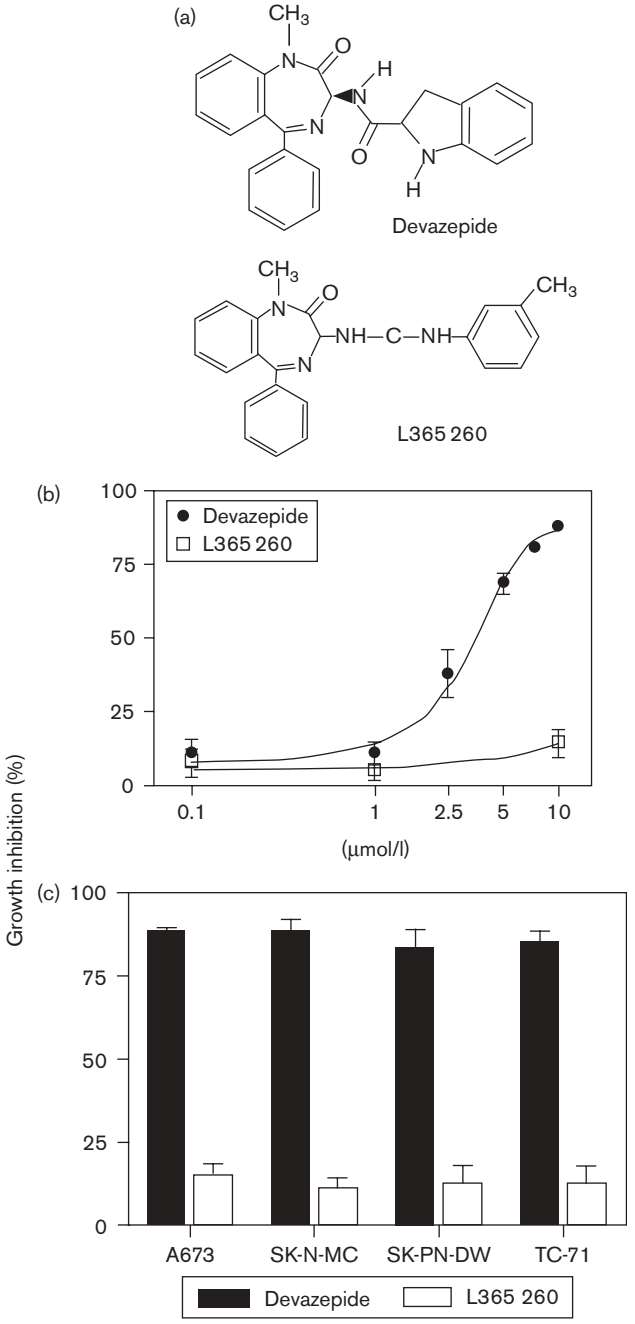
The Student's *t*-test was used for single comparisons of the two groups, whereas two-way analysis of variance using the Student–Newman–Keuls method was used to compare tumor size in control and treated groups of mice. For all analyses, the level of significance was set at P value less than 0.05. All statistical calculations were performed with the GraphPad Prism Version 4.0 statistical software (GraphPad Software, San Diego, California, USA). Data are presented as mean ± standard error.

Results

We have previously shown that CCK acts as an autocrine growth factor in Ewing cells, as silencing CCK in these cells with a specific small interference RNA inhibited cell proliferation and tumor growth *in vivo* [5]. The biological activity of CCK is dependent on its binding to two specific receptors of the seven transmembrane receptor superfamily, the CCK1-R and CCK2-R receptors, which have been shown to be expressed in Ewing tumors and Ewing cell lines [5].

We thus analyzed the effects of CCK receptor antagonists on the proliferation of Ewing cell lines. Two antagonists of CCK receptors were used in these experiments: L364 718 or devazepide, a specific CCK1-R receptor antagonist and L365 260, an antagonist specific for the CCK2-R (Fig. 1a). First, we incubated A673 Ewing cells for 96 h with different concentrations of these antagonists (0.1–10 μmol/l) in presence of serum, and quantify the cell growth by crystal violet staining of the living cells. Devazepide (10 μmol/l) inhibited cell growth of the A673 Ewing cells by nearly 90% when compared with cells incubated with the vehicle alone (the half maximal inhibitory concentration = 3.13 ± 0.52 μmol/l, Fig. 1b). By contrast, the CCK2-R specific antagonist L365 260 had no effect on cell growth at the concentrations tested. Next, we analyzed the effect of both the antagonists on

Fig. 1



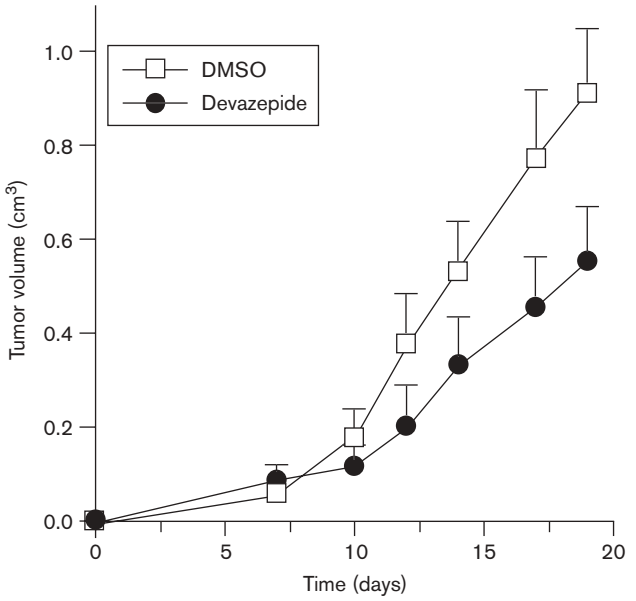
Effects of the CCK1-R receptor antagonist devazepide and the CCK2-R receptor antagonist L365 260 on cell growth of Ewing tumor cells. (a) Chemical structure of the CCK receptor antagonists, devazepide and L365 260. (b) A673 Ewing cells were incubated for 4 days in medium with serum (10%) and in the presence of different doses of devazepide or L365 260 (0.1–10 μmol/l), or with vehicle alone. Afterwards, living cells were stained with crystal violet and the growth inhibition was calculated as the percentage of live cells in the culture with respect to those incubated with vehicle alone. The data shown are the mean ± standard error of 2–6 separate experiments performed in triplicates. (c) Ewing tumor cell lines A673, SK-N-MC, SK-PN-DW, and TC-71 were incubated for 4 days in medium with serum (10%) and 10 μmol/l devazepide or L365 260, or vehicle alone. Growth inhibition was calculated as above and the data shown are the mean ± standard error of 2–8 separate experiments performed in triplicates.

the cell growth in three additional Ewing cell lines to determine whether the effects observed on the Ewing cell lines were generally observed in Ewing cells. Interestingly, devazepide (10 μmol/l) inhibited cell growth to a very similar extent in the four Ewing tumor cells analyzed (range 83.5–88.5%) (Fig. 1c). By contrast, L365 260 (10 μmol/l) had no a significant effect on the growth of Ewing cell lines (range growth inhibition 11.0–15.5%). These data indicate that devazepide inhibits the growth of Ewing tumor cells, while the effect of the specific CCK2-R antagonist L365 260 on cell growth was negligible.

We then assessed whether devazepide was also able to inhibit Ewing tumor growth in-vivo using a nude mouse tumor xenograft model. We injected nude mice with A673 Ewing tumor cells subcutaneously in the left flank of the animals and split the animals into two groups. One group received daily one subcutaneous injection of devazepide in the right flank (10 mg/kg body weight, $n = 10$) and the other one received vehicle alone ($n = 10$). The tumors of the mice treated with devazepide grew significantly more slowly than those of the control group ($P < 0.0023$, two-way analysis of variance, Fig. 2). Moreover, the average tumor volume from animals treated with devazepide was 40% lesser than that in the control group after 19 days of treatment. Remarkably, we observed that one tumor regressed completely upon treatment with devazepide. These data provide evidence that devazepide effectively reduces tumor growth of Ewing cells *in vivo*.

Next, we analyzed the mechanism by which devazepide inhibits growth of Ewing tumor cells. We chose the Ewing cell lines A673 and SK-N-MC for the following experiments. By simple observation under the microscope, we observed that devazepide dramatically increased the number of detached cells in both the Ewing cell lines (Fig. 3). By contrast, cells treated with L365 260 remained attached to the dishes and had the same aspect as control cells exposed to the vehicle alone, which is in agreement with the results shown previously. As these findings suggest that devazepide might induce apoptosis of Ewing tumor cells, we performed flow cytometry analysis on propidium iodine (PI) labeled cells. There was a significant increase in the number of A673 and SK-N-MC cells in the subG_0/G_1 area when they were exposed to devazepide (10 μmol/l) in presence of serum (Fig. 3), which is consistent with an increase in apoptotic cell death. By contrast, L365 260 had no effect on the proportion of cells in the different phases of cell cycle when compared with cells treated with the vehicle alone. When flow cytometry was performed on cells stained with PI and annexin V, the latter detecting the loss of membrane asymmetry and widely accepted as an early marker of apoptosis, three cell populations could be identified (Fig. 4): unlabeled cells (living cells,

Fig. 2



Devazepide inhibits Ewing tumor growth *in vivo*. Nude mice were subcutaneously injected in the left flank with A673 Ewing cells and daily in the right flank with vehicle [dimethyl sulfoxide (DMSO), $n=10$] or devazepide (10 mg/kg body weight, $n=10$). Tumors from the mice that received devazepide grew more slower that those of the control group ($P<0.0023$, two-way analysis of variance).

left-bottom quadrant), cells labeled by annexin V alone (live yet apoptotic cells, right-bottom quadrant), and cells labeled by both annexin V and PI (necrotic cells, right-top quadrant). After 48 h, the proportion of apoptotic cells (annexin V positive/PI negative) increased significantly in cells treated with devazepide. For example, apoptotic cells increased from 10% in SK-N-MC cells treated with the vehicle alone to 32% in cells treated with devazepide. Conversely, 87% of SK-N-MC cells treated with vehicle alone were alive (annexin V labeled/PI negative), whereas this percentage fell up to 55% upon exposure to devazepide. Similar results were obtained with the A673 Ewing cell line. These findings indicate that devazepide induces apoptosis in Ewing tumor cells.

Discussion

Ewing tumors represent a family of very aggressive pediatric malignancies. Even following dose-intensive treatments, 30–40% of patients with localized tumors and 80% of patients with a metastatic disease die through disease progression, making innovative therapies necessary to treat these tumors [1]. Although in recent years important advances have been made in understanding the molecular biology of these tumors, especially in terms of the genes regulated by the specific fusion proteins responsible for the tumors (reviewed in Ref. [4]), few new molecular targets or therapies have been defined. Here, we show that devazepide, a nonpeptide antagonist of CCK receptors, induces apoptosis of Ewing cells and inhibits Ewing cell growth both *in vitro* and *in vivo*.

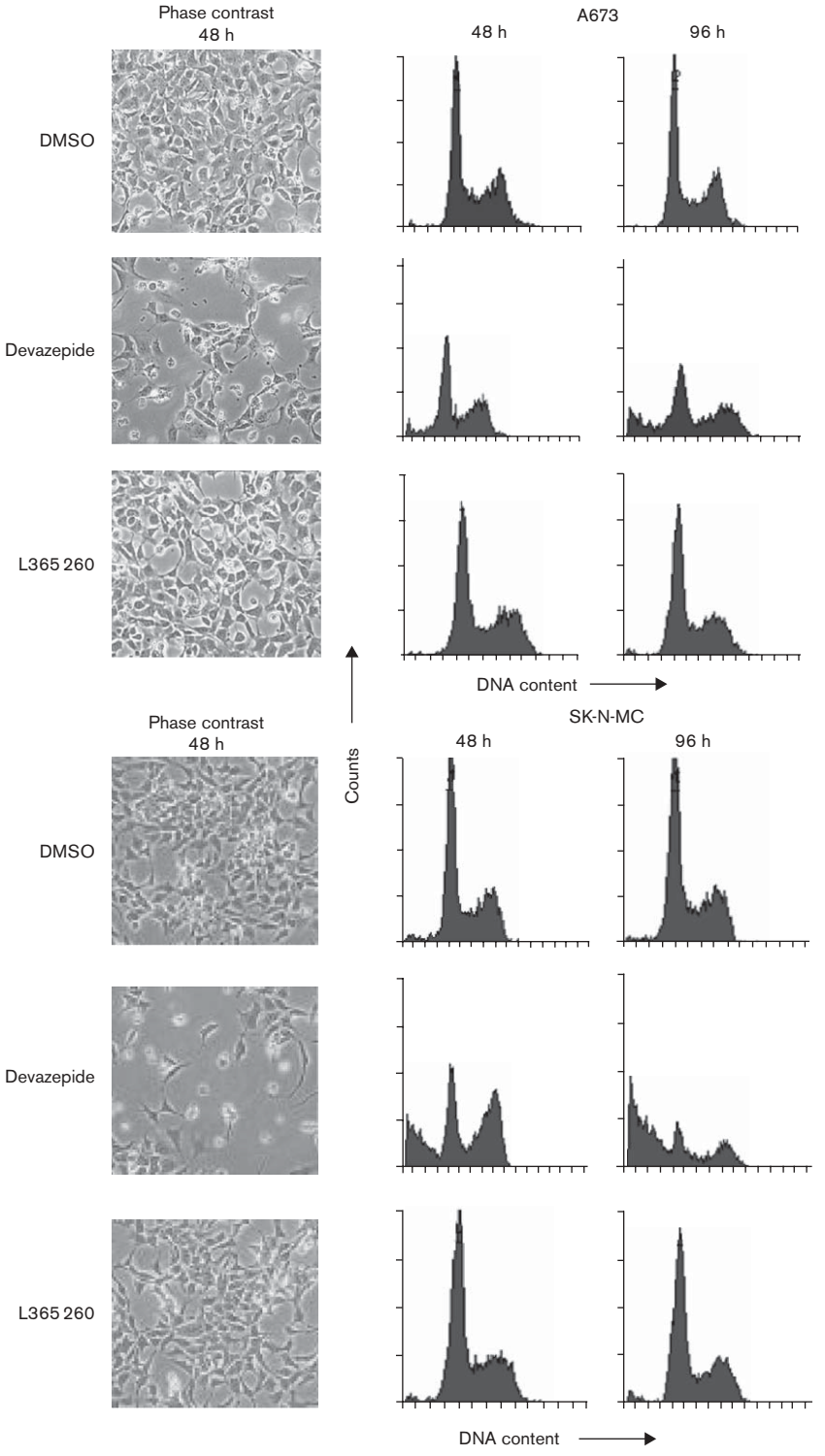
We recently demonstrated that down-regulation of CCK expression by RNA interference impaired Ewing tumor growth *in vivo*, suggesting that CCK is an autocrine growth factor for Ewing tumor cells [5]. Because CCK signals through two well-characterized CCK receptors (namely CCK1-R and CCK2-R), we analyzed the effect of two specific CCK receptor antagonists on cell growth.

We have shown that the CCK1-R receptor antagonist, devazepide, inhibited the growth of Ewing cell lines both *in vitro* and *in vivo*, and also shown that evidence that these effects on cell growth seem to be a consequence of the increased apoptosis induced by devazepide. We also showed that these effects were specific to devazepide, as the CCK2-R antagonist, L365 260, had no significant effect on Ewing cell growth. Several findings indicate that a purely toxic effect of devazepide seems to be unlikely: first, the closely related CCK2-R receptor antagonist L365 260 did not affect cell proliferation in the Ewing cell lines tested; and second, the same doses of devazepide induced only minor growth inhibition in two neuroblastoma cells, suggesting a certain grade of cell specificity (data not shown).

As pointed out above, the rate at which patients with Ewing's sarcoma are cured is poor, particularly those with large or metastatic tumors. Moreover, survival rates have remained stagnant for the past 20 years despite the use of aggressive dose-intensive chemotherapy combined with radiation and surgery. Our data suggest that devazepide could be effective in treating Ewing tumors by inducing the apoptosis of tumor cells. While it is unlikely that devazepide alone will be able to completely eliminate Ewing's tumors, one might expect that in combination with standard chemotherapy, the death of tumor cells could be synergistically increased and the development of resistance diminished by interfering with different pathways essential for tumor cell survival. For example, several in vitro and in vivo studies have shown the efficiency of treatments combining classical chemotherapeutic drugs and inhibitors of autocrine growth factor pathways, such as IGF-1 [13,14]. In this sense, preclinical studies using animal models treated with devazepide in combination with standard chemotherapy agents such as vincristine, actinomycin D, or ifosfamide should be helpfull to determine whether devazepide shows promise as a drug to treat Ewing tumors.

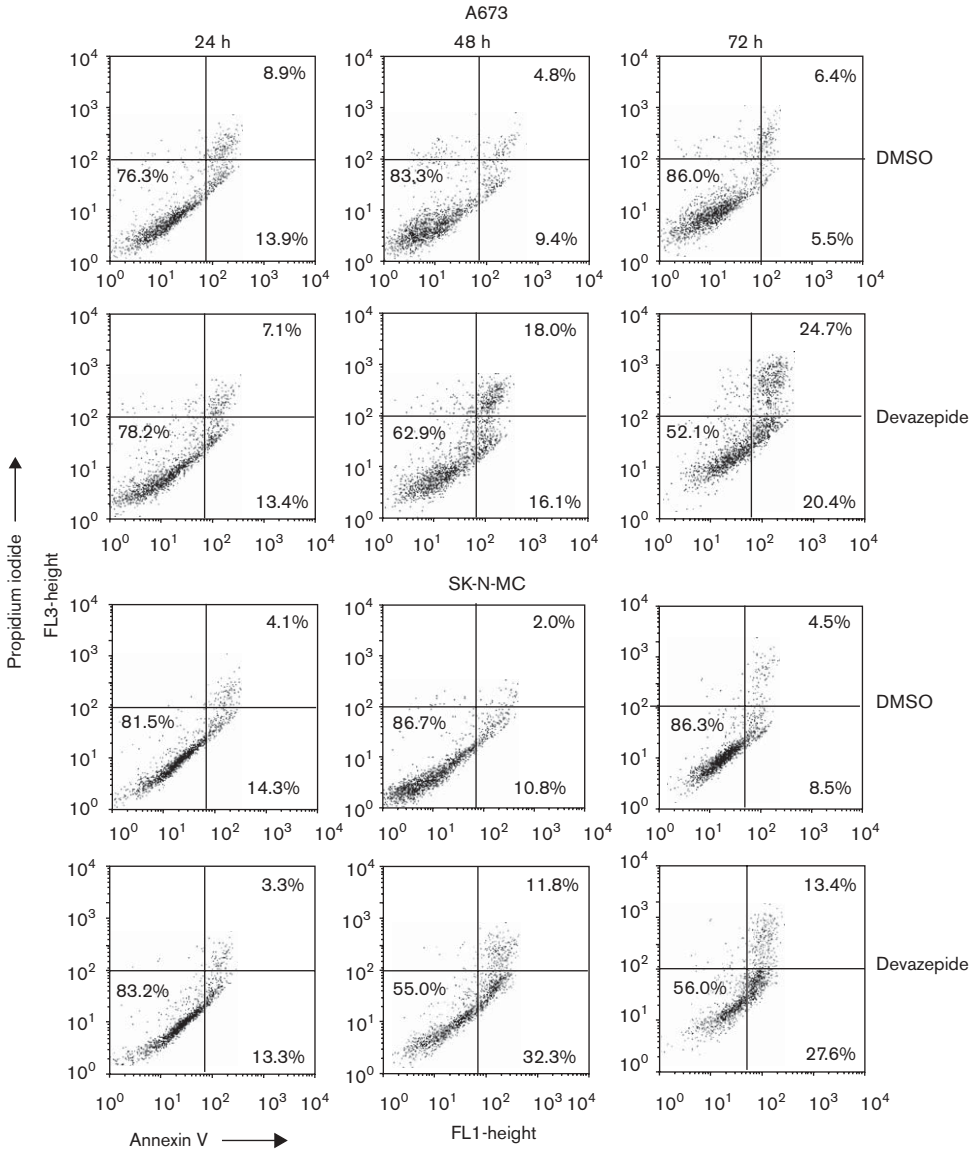
One advantage of devazepide is that its pharmacokinetic and pharmacodynamic properties have been studied in detail. Long-term administration of devazepide is well

Fig. 3



Devazepide induces cell death in Ewing cells. A673 (top) and SK-N-MC cells (bottom) were maintained in medium with serum (10%) and incubated with 10 μmol/l devazepide or L365 260 or vehicle alone (dimethyl sulfoxide, DMSO). After 48 h, numerous detached cells could be observed by phase contrast microscopy in the cultures incubated with devazepide, but not in those incubated with L365 260. Flow cytometry of cells stained with propidium iodide after 48 and 96 h showed that the subG_0/G_1 area, corresponding to dead cells only increased following exposure to devazepide.

Fig. 4



Devazepide induces apoptosis in Ewing cells. A673 (top) and SK-N-MC cells (bottom) were incubated in medium with serum (10%) and 10 μmol/l devazepide or vehicle alone (dimethyl sulfoxide, DMSO) for 24, 48, and 72 h. Flow cytometry of cells stained with propidium iodide and annexin V showed that after 48–72 h in the presence of devazepide, there was a significant increase in apoptotic cells (annexin V positive and propidium iodide negative, right bottom quadrants).

tolerated in different species and it can be administered at doses as high as 2 mg/kg body weight without producing significant side effects [15]. Indeed, short-term administration of devazepide has been tolerated at doses as high as 125 mg/kg without signs of acute toxicity [16]. Devazepide by itself is not nephrotoxic and it is well tolerated in several species, including humans [17,18].

In summary, we have shown that devazepide, a nonpeptide CCK receptor antagonist, induces apoptosis of Ewing tumor cells and inhibits tumor growth in a xenograft mouse model. These findings could contribute to the development of new therapeutic strategies for the treatment of Ewing tumors.

Acknowledgments

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