Abstract. Aim: Several published studies suggest that nonsteroidal anti-inflammatory drugs (NSAIDs) are promising anticancer agents. This study examined the in vitro effect of O-alkylated NSAID hydroxamic acid derivatives 3a-i on cell survival for a panel of human tumour cell lines, their cytotoxicity on normal human fibroblasts and their antiviral activity. Materials and Methods: Established methods of cell viability testings, cell cycle analyses and Western blot assays were used. Results: O-Alkylated NSAID hydroxamic acid derivatives exerted poor antiviral activity but reduced the viability of the studied tumour cell lines in a concentration-dependent manner showing low cytotoxic effect on normal fibroblasts. Compounds 3a and 3i were shown to be potent inhibitors of the growth of MIA PaCa-2 cell line. They induced p53-independent S-phase arrest and triggered caspase 3-dependent apoptosis. Conclusion: Two novel O-alkylated NSAID hydroxamic acid derivatives may be useful in the treatment of pancreatic cancer and should be further evaluated in vivo.

Numerous experimental, epidemiological and clinical studies suggest that nonsteroidal anti-inflammatory drugs (NSAIDs), particularly the highly selective cyclooxygenase (COX)-2 inhibitors, are promising anticancer agents (1). NSAIDs have experimentally been shown to stimulate apoptosis and to inhibit angiogenesis; two mechanisms that help to suppress malignant transformation and tumour growth. A variety of hydroxamic acid derivatives of NSAIDs have been shown to exert efficient anti-inflammatory activities (2, 3). In general, they have a lower acute toxicity, a favourable therapeutic index, are less detrimental to the gastroenteric tract, less irritating and they more easily penetrate through the topical membrane than their respective NSAID precursors (4). In a previous study, NSAID hydroxamic acids demonstrated a strong and non-specific anti-proliferative effect, which was higher than that of their NSAID precursor compounds (5). Among the studied acids, diclofenac and indomethacin hydroxamic acids showed the most pronounced and selective inhibitory activity, while ibuprofen and fenoprofen hydroxamic acids demonstrated higher activity when the hydroxyl group was O-benzyl substituted. The present study focussed on the cytostatic and antiviral activity of O-methyl and O-ethyl NSAID hydroxamic acids and NSAID hydroxamic acids containing free hydroxyl groups, in particular, NSAID hydroxamic acid derivatives 3a-i, containing a metalloproteinase inhibitory moiety and the NSAID pharmacophore.

Materials and Methods

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Key Words: Nonsteroidal anti-inflammatory drugs (NSAID), hydroxamic acids, cytostatic activity, cytotoxicity, human pancreatic cancer, Akt signalling, cell cycle, apoptosis.
(3f), N-ethoxy-2-[2-(2,6-dichlorophenyl)amino]benzenecacetamide (3g), 1-(4-chlorobenzoyl)-2-methyl-N methoxy-5-methoxy-1H indol-3-acetamide (3h) and N-hydroxy-2-(3-benzylophenyl)propanamide (3i). The structures of these compounds are presented in Figure 1.

Chemicals. The following chemicals and reagents were obtained from the indicated companies: Dulbecco’s modified Eagle’s medium (DMEM) (GIBCO BRL, Grand Island, NY, USA); foetal bovine serum (FBS) (Invitrogen, USA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (Sigma Chem Co., St Louis, MO, USA), DC Protein Assay Kit (Bio-Rad, USA), propidium iodide, RNase A and L-glutamine (all from Sigma Chem Co.). Cell cultures were obtained from the American Type Culture Collection (ATCC, USA).

Cell culture. Stock cultures of murine leukaemia (L1210) and murine mammary carcinoma (FM3A) cell lines as well as the human T-lymphocytes (Molt4/C8 and CEM) were cultured as suspension in RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine and 0.075% NaHCO3 in a humidified atmosphere with 5% CO2 at 37°C. Human cervical carcinoma (HeLa), breast carcinoma (MCF-7), metastatic breast adenocarcinoma (SK-BR-3), pancreatic carcinoma (MIA PaCa-2), metastatic colon carcinoma (SW620) and normal human fibroblasts (WI38) were grown as monolayers and maintained in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere with 5% CO2 at 37°C. The non-small cell lung cancer cell line (NCI H727) was cultured in RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, also in a humidified atmosphere with 5% CO2 at 37°C.

Cell viability. Cytostatic activity of the test compounds against L1210, FM3A, Molt4/C8 and CEM cells grown in suspension was measured in 200 μl wells of 96-well microtiter plates (initial cell number: 5–7.5×104 cells/well), as described elsewhere (7). After 48 (L1210, FM3A) or 72 (CEM, Molt4/C8) hours, the tumour cell number was determined by a Coulter counter. In contrast, the panel of the human cervical carcinoma (FM3A) cell lines as well as the human mammary carcinoma (FM3A) cell lines, metastatic breast adenocarcinoma (SK-BR-3), pancreatic carcinoma (MIA PaCa-2), metastatic colon carcinoma (SW620) and normal human fibroblasts (WI38) were grown as monolayers and maintained in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, also in a humidified atmosphere with 5% CO2 at 37°C.

Cell cycle analysis. A total of 2.5×105 cells/well were seeded in 6-well plates. After 24 hours, the cells were treated with the compounds 3a and 3i at concentrations 1×10−6 M and 5×10−6 M. After 24 and 72 hours, the attached cells were trypsinised, combined with floating cells, washed with PBS and fixed with 70% ethanol. Immediately before the analysis, the cells were washed again with PBS and stained with 1 μg/ml of propidium iodide (PI) with the addition of 0.2 μg/ml of RNase A. Cell cycle profiles were obtained using a BD FACSCalibur Flow Cytometer (BD Biosciences, San Jose, CA, USA). Data were analysed using WinMDI 2.9 (http://flowcyt.salk.edu/software.html). Each test point was determined in duplicate in three independent experiments.

Western blot analysis. Untreated cells and cells treated with the compounds 3a and 3i at concentrations 1×10−6 M and 5×10−6 M were lysed after 24 and 72 hours of treatment directly in a buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.2 mM EGTA, 10% glycerol, 1% Triton X-100 and a protease inhibitor cocktail (Roche, Switzerland). Equal amounts of total proteins (40 μg) were resolved on either 12% or 10% SDS-polyacrylamide gels, depending on the molecular mass of the studied protein at constant 100 V and subsequently transferred to a nitrocellulose membrane (Bio-Rad) at constant 200 mA using Mini-PROTEAN Cell (Bio-Rad). The membranes were blocked for 1 hour at room temperature with 4% non-fat dry milk in TBST (50 mM Tris base, 150 mM NaCl, 0.1% Tween 20, pH 7.5). Subsequently, membranes were incubated overnight at 4°C in 3% non-fat dry milk in TBST supplemented with primary antibodies against procaspase 3 (monoclonal anti-procaspase 3 mouse IgG; Pharmingen, BD Biosciences; diluted 1:450), p21 (BD Biosciences; diluted 1:250), p53 (Calbiochem, San Diego, CA, USA; diluted 1:200) and pAkt (monoclonal anti-pAkt mouse IgG; Santa Cruz, CA, USA; diluted 1:200). The membranes were then washed with TBST and incubated for 1 hour at room temperature in TBST containing a secondary anti-mouse antibody linked to horseradish peroxidase. The signal was visualised by the Western Lightening Chemiluminescence Reagent Plus kit (PerkinElmer, USA) on the VersaDoc Imaging System 4000 (Bio-Rad). Signal intensities of the particular bands were measured and compared by the Quantity One software (Bio-Rad). Anti-α-tubulin (Sigma; monoclonal anti-α-tubulin mouse IgG, diluted 1:1000) was used as a loading control.

Antiviral activity assays. The antiviral assays, other than the anti-HIV assays, were based on inhibition of virus-induced cytopathicity in HEL [herpes simplex virus type 1 (HSV-1) (KOS), HSV-2 (G), vaccinia virus and vesicular stomatitis virus], Vero [parafluenza-3, reovirus-1, Sindbis, Coxsackie B4, and Punta Toro virus], HeLa (vesicular stomatitis virus, Coxsackie virus B4, and respiratory syncytial virus), CEF [feline coronavirus (FIV) and feline herpes virus (FHV)] and MDCK [influenza A (H1N1; H3N2) and influenza B] cell cultures. Confluent cell cultures (or nearly confluent for MDCK cells) in microtiter 96-well plates were inoculated with 100 CCID50 of virus (1 CCID50 being the virus dose to infect 50% of the cell cultures). After a 1-hour virus adsorption period, the residual virus was removed, and the cell cultures were incubated in the presence of varying concentrations (100, 20, 4 and 0.8 μM) of the test compounds. Viral cytopathicity was recorded as soon as it reached completion in the control virus-infected cell cultures that were not treated with the test compounds. The methodology of the anti-HIV assays was as follows: human CEM (~3×105 cells/cm2) cells were infected with 100 CCID50 of HIV(IIIb) or HIV-2(ROD)/ml and seeded in 200 μl wells of a
microtiter plate containing appropriate dilutions of the test compounds. After 4 days of incubation at 37°C, HIV-induced CEM giant cell formation was examined microscopically.

**Statistical analysis.** Statistical analysis was performed using Student’s t-test. Tukey’s multiple comparison test was used to determine significant differences among group means (p<0.05).

**Results**

**Effect of O-alkylated NSAID hydroxamic acid derivatives on cell viability.** Firstly, the study examined the effect of O-alkylated NSAID hydroxamic acid derivatives 3a-i on cell survival of the panel of human tumour cell lines and their cytotoxic activity on normal human fibroblasts (WI38) using the MTT assay. O-Alkylated NSAID hydroxamic acid derivatives inhibited the growth of the studied tumour cell lines in a concentration-dependent manner following treatment for 72 hours (Figure 2). Importantly, most of them exerted low cytotoxic effects on WI38 cell cultures (Figure 2 and Table I). Among all compounds tested, the compounds 3h and 3g were the most cytotoxic for WI38 (IC50~36 μM). The compounds 3d, 3g and 3i showed a more pronounced antiproliferative activity on the growth of the two metastatic breast cancer cell lines, MCF-7 and SK-BR-3, than the other compounds. The SK-BR-3 cells overexpress the HER2/c-erb-2 gene product, which is associated with a worse prognosis in patients with breast cancer, perhaps due to an association of the HER-2 proto-oncogene protein with resistance to hormone- and/or chemotherapy. Furthermore, the compounds 3d, 3g and 3h markedly inhibited the growth of metastatic colorectal adenocarcinoma cells SW620. However, no statistically relevant differential effect was observed for these compounds at micromolar concentrations (1 μM and 10 μM).

Interestingly, the in vitro studies of the compounds 3a, 3h and 3i on the human pancreatic cancer cell line MIAPaCa-2 revealed significant cytotoxic activities at low micromolar concentrations with IC50s of 8, 9.7 and 2.2 μM, respectively (Table I). Therefore, these compounds should be considered for further in vivo biological studies (9, 10), especially due to their low cytotoxicity observed on normal human fibroblasts.

**Effect of O-alkylated NSAID hydroxamic acid derivatives on the cell cycle distribution and apoptosis induction.** Due to their strong, highly selective cytostatic effect on MiaPaCa-2 cells (Figure 2 and Table I), the compounds 3a and 3i were chosen for additional mechanistic studies, including cell cycle analysis by flow cytometry and Western blot analysis of the expression of several protein modulators of cell proliferation and death. The flow cytometric analysis showed a marked decrease in the G0/G1 population of the MIA PaCa-2 cells upon 24- and 72-hour treatments with the compounds 3a and 3i at both tested concentrations (1 μM and 5 μM) (Table II), which was accompanied by a clear trend towards accumulation of the cells in S-phase. Interestingly, the treatment of MIA PaCa-2 cells with the compound 3i also resulted in a noticeable increase in G2/M population, especially after 24-hour treatment with the lower tested compound concentration of 1 μM (Table II).

It is well-known that the tumour suppressor gene p53 (TP53) plays a key role in the activation of the G1/S cell cycle checkpoint through its downstream effector, the p21WAF1/Cip1 gene (11). MIA PaCa-2 cells were previously shown to contain a mutated p53 gene (12). The Western blot experiments of the present study also revealed an accumulation of the mutated p53 protein in MIA PaCa-2 cells regardless of treatment conditions, which is non-functional and therefore fails to induce the expression of the p21 protein, as can be seen in Figure 3. These results strongly suggest that the G1-S transition in MIA PaCa-2 cells induced by the treatment with different concentrations of the compounds 3a and 3i occurs independently of p53.

Although the flow cytometric analysis did not reveal a significant increase in the subG0/G1 cell population indicative of apoptotic cells, apoptosis could not be excluded as the mode of MIA PaCa-2 cell death, in response to treatment with the compounds 3a and 3i. Since the procaspase cleavage is a commonly used marker of apoptosis, the ability of the compounds 3a and 3i to promote the cleavage of procaspase-
was measured. In general, caspase-3 exists in a precursor (inactivated) form that is cleaved to generate the active enzyme to act on several death substrates (such as PARP and laminin) to execute apoptosis. Western blot analysis clearly showed a dramatic reduction in the expression levels of procaspase 3 in MIA PaCa-2 cells upon 24- and 72-hour exposure to 5 μM 3a and 3i (Figure 3). When these cells were treated with the lower concentration (1 μM) of the same compounds, the cells initially underwent apoptosis, as evidenced from the diminished expression of the procaspase 3 levels after 24 hours. However, the appearance of a procaspase 3 band after a 72-hour treatment period raises the possibility that a significant fraction of treated MIA PaCa-2 cells survived and escaped from apoptosis.
Activation of Akt (protein kinase B) was previously demonstrated to play a central role in the biology of pancreatic cancer (13, 14). Therefore, the effects of the compounds 3a and 3i on the level of phosphorylated (activated) Akt (pAkt) in MIA PaCa-2 cells were also investigated. Although Akt was reported to be constitutively activated in the majority of human pancreatic cancer cell lines including MIA-PaCa-2 (15, 16), activation of Akt was not observed in MIA-PaCa-2 cells under basal conditions (Figure 3). Specifically, this protein was activated only after a 24-hour exposure to 5 μM 3a and 3i, and to 1 μM 3i. However, prolonged exposure for an additional 48-hour period completely depleted pAkt in MIA-PaCa-2 cells, which points to the abrogation of the Akt signalling pathway.

Antiviral activity of O-alkylated NSAID hydroxamic acid derivatives. The compounds 3a-i were evaluated for their activity against a broad variety of DNA and RNA viruses in cell culture. Unfortunately, none of the compounds showed any pronounced antiviral activity at subtoxic concentrations. Only compound 3i showed marginal activity against the parainfluenza virus, reovirus-1, Punta Toro virus, HSV-1, HSV-2 and vaccinia virus.

Table I. Comparison of IC50 values of the NSAID hydroxamic acid derivatives 3a-i between different tumour cell lines and normal human fibroblasts.

<table>
<thead>
<tr>
<th>Compd.b</th>
<th>L1210</th>
<th>FM3A</th>
<th>Molt4/C8</th>
<th>CEM</th>
<th>HeLa</th>
<th>MCF-7</th>
<th>MIA PaCa-2</th>
<th>NCI-H727</th>
<th>SW620</th>
<th>SK-BR-3</th>
<th>WI38</th>
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<tr>
<td>3a</td>
<td>76±49</td>
<td>218±4</td>
<td>135±5</td>
<td>72±7</td>
<td>&gt;100</td>
<td>66</td>
<td>8</td>
<td>&gt;100</td>
<td>&gt;100</td>
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<tr>
<td>3b</td>
<td>66±21</td>
<td>231±5</td>
<td>94±51</td>
<td>54±10</td>
<td>&gt;100</td>
<td>77</td>
<td>71.8</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>79.1</td>
<td>&gt;100</td>
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<td>3c</td>
<td>38±20</td>
<td>292±105</td>
<td>71±14</td>
<td>45±2</td>
<td>&gt;100</td>
<td>66.8</td>
<td>55.7</td>
<td>&gt;100</td>
<td>39.4</td>
<td>86</td>
<td>&gt;100</td>
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<td>3d</td>
<td>20±4</td>
<td>26±22</td>
<td>35±0</td>
<td>24±12</td>
<td>96.6</td>
<td>25.9</td>
<td>17.7</td>
<td>64.6</td>
<td>23.8</td>
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<tr>
<td>3e</td>
<td>≥ 500</td>
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<td>&gt;500</td>
<td>&gt;100</td>
<td>74.2</td>
<td>&gt;100</td>
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<td>≥ 500</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>&gt;100</td>
<td>96.3</td>
<td>84.8</td>
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<td>97.3</td>
<td>88.2</td>
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<td>3g</td>
<td>34±18</td>
<td>40±4</td>
<td>41±2</td>
<td>25±8</td>
<td>67.9</td>
<td>27.6</td>
<td>53.4</td>
<td>&gt;100</td>
<td>22.7</td>
<td>35.5</td>
<td>36</td>
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<tr>
<td>3h</td>
<td>318±166</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>57.1</td>
<td>38.7</td>
<td>9.7</td>
<td>86.9</td>
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<td>3i</td>
<td>27±17</td>
<td>27±9</td>
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<td>67.1</td>
<td>50.7</td>
<td>21</td>
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*Mean IC50: 50% inhibitory concentration, or compound concentration required to inhibit tumour cell proliferation by 50%. bSee structural formula in Figure 1. Values represent mean±SD of three independent experiments.
However, due to the low antiviral selectivity of this compound, the observed activities may have been due to an underlying cellular toxicity.

**Discussion**

The present study evaluated the ability of the O-alkylated NSAID hydroxamic acid derivatives 3a-3i to suppress the proliferation of different human cancer cell lines along with normal human fibroblasts and assessed the antiviral potency of these compounds. While these compounds exerted a poor, if any, antiviral activity at subtoxic concentrations, they reduced the viability of the studied tumour cell lines in a concentration-dependent manner with a concomitant low cytotoxic effect on normal fibroblasts. These in vitro studies revealed that the compounds 3a, 3h and 3i are potent inhibitors of the growth of the human pancreatic cancer cell line MIA PaCa-2 cells. In general, these compounds gave

![Table III. Cytotoxicity and antiviral activity of compounds 3a-i against several viruses.](image-url)

<table>
<thead>
<tr>
<th>Compd.a</th>
<th>Minimum cytotoxic conc. b (μM)</th>
<th>Parainfluenza-3 virus</th>
<th>Reovirus-1</th>
<th>Sindbis virus</th>
<th>Coxackie virus B4</th>
<th>Punta Toro virus</th>
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<td>&gt;100</td>
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<td>&gt;100</td>
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<tr>
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<td>&gt;20</td>
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</table>

| DS-5000 (μmoles/ml) | >100 | >100 | >20 | >20 | >20 | 20 |

| (S)-DHPA | 250 | 250 | 250 | 250 | 250 |

| Ribavirin | 50  | 146 | 250 | 250 | 50  |

| aSee structural formula in Figure 1; brequired to cause a microscopically detectable alteration of normal cell morphology; crequired to reduce virus-induced cytopathogenicity by 50%. |
rise to a significant decline in G0/G1 phase cells accompanied by an apparent trend towards the accumulation of the cells in S phase under all treatment conditions. In addition, a significant accumulation of G2/M cells was observed after 24-hour incubation with 1 μM 3i. Cell progression from the late G1 into the S phase is regulated in many cells by p53. As MIA PaCa-2 cells lack functional p53 which is incapable of inducing the expression of p21, the G1-S transition in this cell line occurs independently of p53.

The transition from the G1 to the S phase is regarded as a crucial point for deciding between cell growth and apoptosis (17). The Akt (protein kinase B) signalling pathway, a potent crucial point for deciding between cell growth and apoptosis, was previously demonstrated to promote effects of chemotherapy and radiation therapy in a variety of cancer types (18), was previously demonstrated to promote the G1-S transition and to regulate the progression from the G2 to the M phase (19). In view of these findings, the Western blot results of the present study, which revealed the activation of the Akt signalling pathway in the MIA-PaCa-2 cells after a 24-hour exposure to 5 μM compounds 3a and 3i and to 1 μM compound 3i, raised the possibility that this signalling pathway may give cells a growth advantage by promoting the progression from the G1 to the S phase. When these cells were cultured under the same conditions for the next 48 hours, the Akt signalling was abrogated, as confirmed by the complete depletion of pAkt. Therefore, the decreased cell viability observed from the MTT assay after 72 hours could be partially ascribed to the inhibition of the Akt-mediated pro-survival signalling. Similarly, Fahy et al. reported that Akt inhibition is associated with chemosensitisation in MIA-PaCa-2 cells (16).

Apoptosis is a cell suicide programme characterized by unique cellular events such as mitochondrial fragmentation and dysfunction, nuclear condensation, cytoplasmic shrinkage and activation of apoptotic protease caspases. The compounds 3a and 3i triggered caspase 3-mediated apoptosis in the MIA-PaCa-2 cells at 5 μM concentration, regardless of the treatment period, as evidenced by the reduction in the expression levels of procaspase-3. However, when the cells were exposed to a lower concentration of these two compounds (1 μM), they initially underwent apoptosis, but a significant portion of the cells managed to survive by escaping from apoptosis, as confirmed by the expression of procaspase-3 that resumed almost to a basal level after 72 hours. Similarly, Tang et al. showed that cancer cells could survive after the initiation of the apoptosis induced by different stimuli and the reversibility of apoptosis was demonstrated in various cancer cell lines (20). Interestingly, the cancer cells were able to escape from apoptosis in spite of ongoing critical apoptotic events, including the activation of caspase-3.

The cellular mechanisms that switch between survival and death could be regulated at different molecular levels, including the activation of caspase-3. This death protease controls the level of its own activation by targeting RasGAP, a caspase substrate that harbours two cleavage sites used sequentially as caspase activity increases in cells (21). The first cleavage of RasGAP occurs when caspase-3 is mildly activated, generating an N-terminal fragment called fragment N that activates Akt and promotes cell survival in stress or adverse conditions (21). At higher caspase activity, RasGAP is further cleaved at position 157, leading to a marked reduction in Akt activity and induction of the execution phase of apoptosis (22). Therefore, RasGAP functions as a sensor of caspase activity to determine whether or not a cell should survive (23). Based on these findings and the Western blot results of the present study, it seems reasonable to conclude that activation of caspase-3 and Akt activation/inhibition are tightly connected events that determine the mode of MIA-PaCa-2 cell response to the compounds 3a and 3i.

Conclusion

O-Alkylated NSAID hydroxamic acid derivatives 3a and 3i exerted potent anti-proliferative effects on MIA-PaCa-2 cells. These compounds induced p53-independent S-phase arrest with concomitant decline in G0/G1 phase cells. In addition, they triggered a caspase-3-dependent apoptosis in time- and concentration-dependent manner. The inhibition of the pro-survival Akt signalling pathway by the compounds 3a and 3i after a 72-hour exposure could be associated with decreased cell viability as revealed by the MTT assay.

In summary, considering the low cytotoxic effects of the compounds 3a and 3i on normal human fibroblasts and their ability to specifically abrogate the Akt signalling pathway, which is considered an important molecular factor in the pathogenesis of human pancreatic cancer, these two novel O-alkylated NSAID hydroxamic acid derivatives may be useful in the treatment of pancreatic cancer and should be further evaluated in vivo.

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