Valproic acid, atorvastatin and pioglitazone belong to different therapeutic drug classes and exhibit biological activities that are capable of interfering with cancer development. In this study, we examined the anticancer synergistic effects of these three drugs in experimental models in vitro and in vivo. Cytotoxic activity was determined against K562, NCI-H292 and HEp-2 lines cells. The Ehrlich carcinoma was used as in vivo model. Atorvastatin associated with pioglitazone, presented cytotoxicity against NCI-H292 cells with IC_{50} value 3.75 µg/ml. The ultra-structural analysis showed that the atorvastatin in combination with pioglitazone induced apoptosis in 66.3% of the cells. Treatment with either valproic acid or valproic acid + atorvastatin + pioglitazone presented cytotoxicity in Ehrlich carcinoma cells, with IC_{50} values equal to 10.8 and 11.4 µg/ml, respectively. In evaluation of antitumor effects in vivo it was observed that valproic acid or the atorvastatin + pioglitazone induced tumor inhibition of 60.2 and 64.9%, respectively. However, histopathology analysis suggests that the liver and kidneys are affected by both treatments. In conclusion, the data indicate that atorvastatin + pioglitazone present synergistic anticancer effect in lung cancer cells and solid tumours.

Key words: Anticancer, atorvastatin, Ehrlich carcinoma, thiazolidinedione.

INTRODUCTION

Valproic acid (VPA), atorvastatin (ATOR) and pioglitazone (PIO) are drugs trade belonging to different therapeutic classes and are used widely in the world. The main indication for the use of VPA is the treatment of epileptic seizures, but it is also a histone deacetylase inhibitor (HDACi). Atorvastatin belongs to the class of statins, used mainly in the cholesterol control by inhibiting the 3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoA). Pioglitazone is a thiazolidinedione (TZD) with hypoglycemic action. This class of molecules is peroxisome proliferator-activated receptor gamma (PPARγ) agonist. The potential of histone deacetylase inhibitors, statins and PPARγ agonists have been investigated as an alternative in treatment of various cancers (Marchion and Münster, 2007; Mrówka et al., 2008; Sassano and Platanias, 2008).

Post-translational histone modifications such as acetylation are associated with transcriptionally active
regions of the genome. HDACis possess antitumor activities and have the potential to induce re-expression of genes abnormally suppressed in cancer cells, thus potentially inducing growth arrest, differentiation, and/or apoptotic cell death of transformed cells in vitro and in vivo (Bolden et al., 2006; Marks, 2010). Several HDACi are currently undergoing clinical testing and recently some have been approved for the treatment of cutaneous T-cell lymphoma (Munster et al., 2009). However, some research indicates that anticancer effects induced by these drugs may be more effectively exploited when used in combination with others chemotherapeutics (Ramalingam et al., 2007).

The anticancer effect of statins is the subject of numerous investigations, several results have reported efficacy of HMG-CoA reductase inhibitors against various types of cancer as leukemia, prostate, lung, colorectal and breast (Sławińska and Kandefer-Szmerszeń, 2008; Bardou et al., 2010; Papadopoulos et al., 2011). PPARγ agonists demonstrate mechanisms of suppression of tumor development in several in vitro and in vivo models. The proposed mechanisms for the antitumor effects of TZDs, apoptosis induction, cell cycle arrest, and differentiation have been extensively reported (Grommes et al., 2006; Blanquicett, 2008; Lichtor et al., 2008).

The concept of using a combination of agents for cancer chemoprevention or treatment has recently received much attention. Combinations of two or more agents that have different mechanisms of action have been suggested as a promising strategy to maximize efficacy and minimize toxicity (Lu et al., 2008). Although pre-clinical studies exist in the literature for ATOR, PIO and VPA, there are no reports of the anticancer effects induced by these drugs combined.

MATERIALS AND METHODS

Drugs and chemicals

Atorvastatin was obtained from LKT Laboratories, Inc., St. Paul, MN, USA. Valproic acid, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT), L-glutamine and doxorubicin were purchased from Sigma Aldrich Co., St Louis, MO, USA. Dulbecco’s Modified Eagle Medium (DMEM), fetal bovine serum, penicillin (Pen) and streptomycin (Strep) were purchased from Gibco. Pioglitazone was obtained from Takeda Pharmaceuticals, Inc. (Pen) and streptomycin (Strep) were purchased from Gibco. Modified Eagle Medium (DMEM), fetal bovine serum, penicillin, and streptomycin (Strep) were purchased from Gibco. 2H tetrazolium bromide (MTT), L-glutamine, and doxorubicin were obtained from Sigma Aldrich Co., St Louis, MO, USA. Pioglitazone was obtained from Takeda Pharmaceuticals, Inc., Deerfield, IL, USA.

Animals

A total number of 72 Swiss albino mice (male, 25 to 30 g), obtained from the animal house of Antibiotics Department of the Federal University of Pernambuco, Brazil, were used. The animals were kept in cages with free access to food and water, under 12 h light-dark cycles. The animals were treated in accordance with the International Council for Laboratory Animal Science (ICLAS), and following the ethical principles of the Brazilian Society of Science in Laboratory Animals (SCBAL). All experiments were approved by the Ethics Committee for Animal Experimentation of the Biological Sciences Center of the Federal University of Pernambuco, Brazil, number 23076.002926/2009-06.

Cells

The cytotoxicity of the drugs and drug combinations was tested against the following cell lines: K562 (human erythroleukemia), NCI-H292 (human lung mucoepidermoid carcinoma), HEP-2 (human laryngeal epidermoid carcinoma) and Ehrlich carcinoma (murine breast adenocarcinoma). The cells were grown in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 μg/ml Strept and 100 U/ml Pen, and incubated at 37°C in a 5% CO2 atmosphere.

Human cells were obtained from the Adolfo Lutz Institute, São Paulo, Brazil. Ehrlich carcinoma cells were maintained in the peritoneal cavities of the male Swiss mice in the Bioassays Laboratory for Pharmaceutical Research, Antibiotics Department from the Federal University of Pernambuco.

Cytotoxic activity

The cytotoxicity was evaluated by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (Mosmann, 1983). The tumor cell lines were seeded into 96-well plates (105 cells/well for adherent cells or 0.5 × 105 cells/well for suspension cells; 100 μl of medium). After 24 h, drugs or drug combinations (0.9 to 25 μg/ml) were added to each well and incubated for another 24 h at 37°C in 5% CO2. Doxorubicin was used as positive control. After 72 h, 10 μl of MTT solution (5 mg/ml) was added to each well and incubated for 3 h. At the end of this period, the medium was discarded and formazan crystals were dissolved in 100 μl of DMSO. The absorbance of each sample was spectrophotometrically measured at 450 nm with a micro plate reader (Model 3550 BIO-RAD, Inc).

Transmission electron microscopy (morphological detection of apoptosis)

For morphological assessment of apoptotic cell death, transmission electron microscopy was used for the NCI-H292 cell line, which showed the lowest IC50 values for ATOR and ATOR + PIO. Cells were treated for 72 h and were later fixed with 2.5% glutaraldehyde (Sigma) and 4% paraformaldehyde (Sigma) in 0.1 M cacodylate buffer. After fixation, the samples were washed twice in the same buffer and post-fixed in 0.1 M cacodylate buffer (pH 7.2) containing 1% osmium tetroxide (Sigma), 2 mM calcium chloride and 0.8% potassium ferricyanide. Next, cells were dehydrated using acetone and embedded in SPIN-PON resin (Embed 812). Polymerization was performed at 60°C for three days. Ultrathin sections were collected on nickel 300-mesh grids, counterstained with 5% uranyl acetate and lead citrate and examined with an FEI Morgani 268D transmission electron microscope. A minimum of 40 cells per sample were observed from three independent experiments to evaluate any cellular morphological alterations (Ma et al., 2007).

In vivo antitumor activity

Ehrlich ascites carcinoma (EAC) cells were derived from a spontaneous murine mammary adenocarcinoma. EAC cells were maintained in the undifferentiated form by passaging in syngeneic mice by transplanting 25 × 106 cells/ml (i.p.) each week. The ascitic fluid was removed by opening the belly and collecting all of the fluid with a sterile syringe. Ascitic tumor cell counts were carried out using the trypan blue dye exclusion method with a Neubauer
hemocytometer. Animals received 200 µl of a suspension containing 5 × 10⁶ cells/ml (i.p.) as described previously (Matsuzaki et al., 2003). Treatment began 24 h after tumor cell inoculation and was administered once daily in a single dose for 7 consecutive days. In the treatments with isolated drugs, they were used in therapeutic doses, drug combinations were defined by dose response curve in vitro. The doses of each drug, drug combinations and treatment controls are listed in Table 1. For the negative control group, a 0.9% solution of NaCl was used. On the ninth day, the mice were sacrificed and the tumors were dissected, weighed and fixed in 10% formaldehyde. The inhibition rate (%) was calculated as follows: inhibition rate (%) = [(A – B) A] × 100, where A is the average weight of the negative control group and B is the average tumor weight of the treated group (Bezerra et al., 2008).

Toxicological analysis

Animals bearing tumors were observed daily throughout the experiment in order to identify any adverse reactions or abnormal signs. The body mass was measured at the beginning and end of the treatment regime. At the end of treatment, animals were anesthetized with ketamine and xylazine and blood samples were collected for hematological analysis. After sacrifice, the animals were autopsied to observe the internal organs with respect to position, form, size and color in order to describe signs of grave lesions. The kidneys, liver and spleen were removed, weighed and fixed in 10% formaldehyde.

Hematological analysis was carried out using an automatic cell counter (ABX-MICROS-60 cell counter Horiba, Inc). The samples were evaluated for the following hematological parameters: number of erythrocytes, concentration of hemoglobin, number of platelets and total count and differential of leukocytes.

Histopathological analyzes

After being fixed in formaldehyde, the tumors, livers, spleens and kidneys were put into paraffin. The blocks were cut using a microtome to a thickness of 4 µm, and the slides were stained with hematoxylin and eosin for morphological analysis. Histological analysis was performed by light microscopy in order to recognize any alterations attributed to the drugs.

Calculations for the effects of drug combinations

The effects of drug combinations on the inhibition of cell growth were calculated using the combination index (CI), which is designed for drugs with "mutually nonexclusive" mechanisms of action (Chou, 1996; Reynolds and Maurer, 2005).

<table>
<thead>
<tr>
<th>Drug</th>
<th>Groups and doses (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>NaCl 0.9%</td>
<td>-</td>
</tr>
<tr>
<td>Valproic acid</td>
<td>-</td>
</tr>
<tr>
<td>Atorvastatin</td>
<td>-</td>
</tr>
<tr>
<td>Pioglitazone</td>
<td>-</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 1. Drug doses in mg/kg and drug combinations used for each experimental group.

Statistical analysis

The results are presented as the mean ± standard deviation (SD). The differences among the experimental groups were compared using an analysis of variance (ANOVA), followed by a Student Newman-Keuls test. The level of significance adopted in the tests was 5% (p<0.05).

RESULTS

The cytotoxic activity of VPA, ATOR and PIO, alone and in various combinations, was tested against three human cancer cell lines as well as the murine Ehrlich carcinoma cell line (Table 2). ATOR and ATOR+PIO showed significant cytotoxicity against NCI-H292 cells with IC₅₀ values of 5.54 and 3.75 µg/ml, respectively. The CI for ATOR + PIO in this cell line was 0.57, indicating that this drug combination promotes synergistic effects.

No significant results were obtained from the cell lines HEP-2 or K562. VPA and ATOR + PIO showed significant cytotoxicity towards EAC cells with IC₅₀ values of 10.8 and 11.4 µg/ml, respectively. The CI value for the ATOR + PIO combination was 0.87, suggesting slight synergism. The doxorubicin positive control exhibited IC₅₀ values statistically significant against all the cell lines studied.

Transmission electron microscopy is the best method for observing morphological changes in the cytoplasm and nucleus of cells and can delineate the apoptosis process with great precision. Figure 1A shows the typical morphology of control cells (treated with DMSO 0.1%) where 88 ± 3.7% showed a complete surface of perfect clarity with the nuclear membrane and mitochondria in good shape. The ultrastructure of NCI-H292 cells treated with ATOR showed intense cytoplasmic vacuolization in 43.4 ± 6.3% of the cells and 26.2 ± 4.8% of chromatin condensation at the perinuclear margin (Figure 1B).

The cells treated with ATOR+PIO and doxorubicin showed typical apoptotic morphology in 66.3 ± 3.8 and 71.4 ± 2.3% of cells, respectively, beyond the stage of cytoplasmic and nuclear degeneration (Figure 1C and D). The effects of VPA, ATOR and PIO, alone and in various combinations, against Ehrlich carcinoma are described as shown in Figure 2. All of the treated groups showed tumor inhibition with respect to the control group
Table 2. The effect of individual drugs and drug combinations on human cancer cell lines and Ehrlich carcinoma.

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC₅₀ (µg/ml)</th>
<th>NCI-H292</th>
<th>HEp-2</th>
<th>K562</th>
<th>Ehrlich carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Valproic acid (VPA)</td>
<td>&gt;25</td>
<td>&gt;25</td>
<td>&gt;25</td>
<td>&gt;25</td>
<td>10.8 (10.1 – 11.49)</td>
</tr>
<tr>
<td>Atorvastatin (ATOR)</td>
<td>5.54 (5.27 – 5.83)</td>
<td>&gt;25</td>
<td>&gt;25</td>
<td>&gt;25</td>
<td>24.79 (22.11 – 27.05)</td>
</tr>
<tr>
<td>Pioglitazone (PIO)</td>
<td>&gt;25</td>
<td>&gt;25</td>
<td>&gt;25</td>
<td>&gt;25</td>
<td>&gt;25</td>
</tr>
<tr>
<td>VPA + ATOR</td>
<td>15.26 (14.58 – 16.15)</td>
<td>&gt;25</td>
<td>&gt;25</td>
<td>&gt;25</td>
<td>23.6 (22.08 – 25.12)</td>
</tr>
<tr>
<td>VPA + PIO</td>
<td>&gt;25</td>
<td>&gt;25</td>
<td>&gt;25</td>
<td>&gt;25</td>
<td>&gt;25</td>
</tr>
<tr>
<td>ATOR + PIO</td>
<td>3.75 (3.57 – 3.92)</td>
<td>23.02 (22.12 – 24.26)</td>
<td>&gt;25</td>
<td>&gt;25</td>
<td>11.39 (10.31 – 12.47)</td>
</tr>
<tr>
<td>VPA + ATOR + PIO</td>
<td>&gt;25</td>
<td>&gt;25</td>
<td>&gt;25</td>
<td>&gt;25</td>
<td>&gt;25</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>4.89 (3.98 – 5.69)</td>
<td>5.29 (4.12 – 6.31)</td>
<td>3.21 (2.93 – 3.99)</td>
<td>4.77 (2.33 – 7.41)</td>
<td></td>
</tr>
</tbody>
</table>

The data are presented as IC₅₀ values with 95% confidence interval from three independent experiments, carried out in quadruples.

Figure 1. Transmission electron micrograph of NCI-H292 cells. (A) DMSO control (x5600): normal cell morphology; (B) ATOR (x7500): chromatin condensation at the perinuclear margin and many vacuoles; (C) ATOR + PIO (x7100): apoptotic cell with intense chromatin condensation and cytoplasmic degeneration; (D) Doxorubicin (x7100): apoptotic cell with complete degeneration of the nuclear membrane, chromatin condensation and cytoplasmic vacuoles.

(p<0.05). At the end of the experiment, the mean mass of the tumors from the control group was 2.43 ± 0.17 g. In the animals treated with VPA, the weight of the Ehrlich carcinoma was reduced to 0.96 ± 0.13 g; in the group treated with ATOR + PIO, the tumor mass was reduced to 0.9 ± 0.06 g. The CI value for ATOR + PIO combination was 0.71, indicating a synergistic mechanism of action between the two drugs. In the group treated with doxorubicin, the average tumor weight was reduced by 70.5%.

Histopathology analysis of the negative control tumors showed pleomorphic polygonal cells that were hyperchromatic and binucleated. Various degrees of nuclear and cellular pleomorphism were observed (Figure 3A). In the animal groups treated with VPA or ATOR + PIO muscular tissue invasion and extensive areas of coagulating necrosis
Figure 2. The effect of VPA, ATOR and PIO, alone and in combination, in mice transplanted with Ehrlich carcinoma. Values are presented as the mean ± the standard deviation, where a = (p <0.05) compared with the control by ANOVA followed by the Student Newman-Keuls post-test.

Figure 3. Photomicrograph showing the histopathology evaluation of the Ehrlich carcinoma from animals treated with 0.9% NaCl (A), 300 mg/kg VPA (B), 5 mg/kg ATOR + 7.5 mg/kg PIO (C) and 5 mg/kg doxorubicin (D) analyzed with a light microscope (x400).

necrosis were observed (Figure 3B and C). The group treated with doxorubicin showed invasion of adipose tissue (Figure 3D).

In the animals treated with VPA or ATOR+PIO, there was an increase in body weight; the mass gain was significantly different from the control and other groups (p < 0.05). In the group treated with doxorubicin, there was a significant reduction of body mass with respect to the weight; these results were significantly different than the control (p < 0.05).

Upon analyzing the blood from mice with Ehrlich carcinoma, statistically significant alterations were
Figure 4. The effect of AVP and the ATOR + PIO combination treatment on the liver of mice transplanted with Ehrlich carcinoma. Photomicrography showing the histopathology evaluation of the livers of animals treated with saline (0.9% NaCl) (A), 300 mg/kg VPA (B), 5 mg/kg ATOR + 7.5 mg/kg PIO (C) and 5 mg/kg doxorubicin (D) analyzed by light microscopy (x400). The thin arrow indicates the hydropic degeneration of hepatocytes and the large arrow indicates venous congestion.

those treated with VPA or doxorubicin (Table 3). Treatment with VPA induced greater thrombocytopenia versus the control and other experimental groups (p < 0.05). Those animals treated with doxorubicin presented a reduction in the total number of platelets and leukocytes compared to the control and other treatment groups (p < 0.05). The hematologic toxicity observed in this study are adverse reactions provoked by the drugs, mainly inhibiting bone marrow activity (Al-Harbi et al., 1992).

Histopathology analysis of the livers of animals in the negative control group presented well-preserved parenchyma, with morphologically regular hepatic cells constituting organized hepatic lobules. The hepatic sinusoids did not present abnormalities and the Kupffer cells appeared in normal quantities (Figure 4A). The animals treated with VPA and ATOR+PIO presented hydropic degeneration of hepatocytes and venous congestion (Figures 4B and 4C). The kidneys of the group treated with VPA did not reveal any relevant histological alterations compared with the negative control (Figure 5A and B). In the kidneys of animals treated with ATOR+PIO, some necrotic glomeruli and other signs indicative of partial necrosis of glomeruli were observed (Figure 5C).

The spleens of the animals treated with VPA or ATOR+PIO did not show relevant histological alterations in relation to the negative control. The animals treated with doxorubicin (Figures 4D and 5D) did not present relevant histopathology alterations in any of the organs evaluated in this study.

DISCUSSION

This paper presents new results regarding the use of combinations of drugs that have different mechanisms of action. The synergistic effect of the ATOR+PIO combination stood out among our results; this combination induced apoptosis to cancer cell lines in vitro and revealed a prominent reduction in tumor growth in vivo.

In the human lung mucoepidermoid carcinoma cell line, treatment with ATOR or ATOR+PIO showed high cytotoxicity. The combination of ATOR+PIO produced a synergistic effect, inducing apoptosis in NCI-H292 cells. Moreover, the combination of these drugs displayed synergistic mechanisms of cytotoxicity in vitro and to Ehrlich tumor reduction in vivo. According Kaufman and Chabner (2001), drug combinations are especially effective when the additive or synergistic effects increase cytotoxicity for tumor cells while reducing adverse effects. Virtually all curative chemotherapy regimens employ multidrug combinations. However, the vast majority of those combinations have been developed empirically. Several studies have demonstrated the pro-apoptotic action of statins, which could relate to the decreased expression of anti-apoptotic proteins such as Bcl-2.
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Figure 5. The effect of VPA and the ATOR + PIO combination on the kidneys of mice transplanted with Ehrlich carcinoma. Photomicrography showing the histopathology evaluation of the kidneys from animals treated with 0.9% NaCl (A), 300 mg/kg VPA (B), 5 mg/kg ATOR + 7.5 mg/kg pioglitazone (C) and 5 mg/kg doxorubicin (D) analyzed by light microscopy (x400). The thin arrow indicates necrotic glomeruli and the large arrow indicates partial necrosis of the glomerulus.

(Dimitroulakos et al., 2000), the activation of caspases 3, 7, 8 and 9 (Cafforio et al., 2005), induced apoptosis via the suppression of ERK1/2 and Akt activation (Yanae et al., 2011) and the reactivation JNK, which is a kinase that can act as a tumor suppressor (Meral et al., 2007). Some pre-clinical studies have also shown that statins have a capacity to induce synergistic antitumor effects associated with conventional chemotherapeutics (Gan et al., 2008; Martirosyan et al., 2010; Chen et al., 2013).

PPARγ is ligand-dependent nuclear transcription factor that is highly expressed in adipocytes. PPARγ agonists have been demonstrated to inhibit cell proliferation and may act on cell cycle control check points (Koeffler, 2003; Heaney, 2004). The action of PPARγ is also related to apoptosis, principally by reducing the gene expression of BCl2 and increasing the expression of p27, c-myc, BAX and BAK (Ohta et al., 2001; Martelli et al., 2002; Li et al., 2003). Pioglitazone treatment has induced apoptosis and a notable decrease in cell proliferation in leukemia cell lines and in colon cancer cells (Zang et al., 2006; Cerbone et al., 2007). The combination of ATOR + PIO or other statins and thiazolidinediones has shown synergic interactions towards the treatment and prevention of cardiovascular illnesses and diabetes through diverse experimental models (Forst et al., 2007, 2008; Hanefeld et al., 2007). Our results showed that ATOR+PIO inhibited proliferation of lung mucoepidermoid carcinoma; other studies also reported the anticancer effect of HMG-CoA inhibitor and PPARγ agonist in lines of lung cancer (Li et al., 2010; Chen et al., 2012, 2013). Treatment with ATOR+PIO did not induce alterations in any measured hematological parameters, whereas histopathology analysis of the liver and kidneys revealed signs of toxicity, considered an adverse reaction knowingly associated with the metabolism of drugs used (Farley-Hills et al., 2004).

In this study, VPA induced considerable cytotoxicity in vitro and was able to inhibit the Ehrlich tumors in vivo. Our results corroborate those from other studies that demonstrate the effectiveness of VPA against diverse types of cancer. A reduction in tumor cell proliferation, an inhibition of angiogenesis, the promotion of apoptosis and provocation of cellular differentiation in vitro and in vivo have all been observed (Liu et al., 2006).

Several authors have adopted the hypothesis that VPA acts principally by inducing apoptosis and differentiation in various types of cancerous cells (Valentini et al., 2007). Experiments have demonstrated the efficacy of VPA in combination with other drugs in inducing apoptosis and in potentiating the activity of the combined drugs (Frew et al., 2008; Munster et al., 2009; Xie et al., 2012). Our results revealed alterations in body mass, in hematological parameters and in hepatic toxicity induced by VPA. Devane (2003) attributes these physiological effects to the adverse reactions from VPA.

Conclusively, this study shows the promising potential of the drug combination ATOR + PIO. Until now, there have been no reports describing the therapeutic
combination therapeutic combination of these drugs and their mechanisms of action. Our results reinforce the anticancer potential of valproic acid, atorvastatin and pioglitazone, demonstrating tumor growth inhibition in vivo with insignificant changes to the blood parameters or morphological and histopathological features.

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