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Anti-MDR and antitumoral action of acetylsalicylic acid on leukaemic cells

Michele CARRETT-DIAS*, Ana Paula de Souza VOTTO*, Daza de Moraes Vaz Batista FILGUEIRA*, Daniela Volcan ALMEIDA*, Adriana Lima VALLOCHI†, Marcelo Gonçalves Montes D'OCA‡, Luis Fernando MARINS*§ and Gilma Santos TRINDADE*§[1]

*Programa de Pós-graduação em Ciências Fisiológicas – Fisiologia Animal Comparada, Universidade Federal do Rio Grande (FURG), 96201-900 Rio Grande, RS, Brazil, †Fundação Oswaldo Cruz, Instituto Oswaldo Cruz, 21045-900 Rio de Janeiro, RJ, Brazil, ‡Escola de Química e Alimentos, FURG, 96201-900 Rio Grande, RS, Brazil, and §Instituto de Ciências Biológicas, FURG, 96201-900 Rio Grande, RS, Brazil

Synopsis

ASA (acetylsalicylic acid) is an NSAID (non-steroidal anti-inflammatory drug). ASA has gained attention as a potential chemopreventive and chemotherapeutic agent for several neoplasms. The aim of this study was to analyse the possible antitumoural effects of ASA in two erythroleukaemic cell lines, with or without the MDR (multidrug resistance) phenotype. The mechanism of action of different concentrations of ASA were compared in K562 (non-MDR) and Lucena (MDR) cells by analysing cell viability, apoptosis and necrosis, intracellular ROS (reactive oxygen species) formation and *bcl-2*, *p53* and *cox-2* gene expression. ASA inhibited the cellular proliferation or induced toxicity in K562 and Lucena cell lines, irrespective of the MDR phenotype. The ASA treatment provoked death by apoptosis and necrosis in K562 cells and only by necrosis in Lucena cells. ASA also showed antioxidant activity in both cell lines. The *bcl-2*, *p53* and *cox-2* genes in both cell lines treated with ASA seem to exhibit different patterns of expression. However, normal lymphocytes treated with the same ASA concentrations were more resistant than tumoral cells. The results of this work show that both cell lines responded to treatment with ASA, demonstrating a possible antitumoral and anti-MDR role for this drug.

Key words: acetylsalicylic acid (ASA), antioxidant, death cell, gene expression, leukaemia, multidrug resistance (MDR)

INTRODUCTION

ASA (acetylsalicylic acid or aspirin) is an NSAID (non-steroidal anti-inflammatory drug) that is used clinically for its anti-inflammatory, antipyretic and analgesic properties. ASA and other NSAIDs have gained attention as potential chemopreventive agents for several neoplasms, including colorectal [1], endometrial [2], ovarian [3], oesophageal [4], lung and breast cancers [5].

Besides the conventional tools for cancer treatment, which include: radiotherapy, chemotherapy and surgery, other alternative therapies have been proposed, e.g., photodynamic therapy, employing a photosensitizing agent and different wavelengths of non-ionizing radiation and oxygen [6,7]. Additionally, ASA has been indicated as another method to combat cancer.

ASA is known to inhibit the cyclo-oxygenases, Cox-1 (constitutive isoform) and Cox-2 (induced isoform). These Cox enzymes have been implicated in carcinogenesis through the production of PGs (prostaglandins) and a decreased cancer risk may be attributable to the inhibition of PG synthesis, enhancement of the cellular immune response or induction of apoptosis [8]. In such cases, it is reasonable to surmise that PG production is inhibited along with the inhibition of the Cox enzyme, providing an inhibition of the processes of cancer formation.

The antimutagenic and antioxidant properties of NSAIDs have been investigated. The suppressive and protective effects of ASA as an antitumoral drug might be at least partially ascribed to its antioxidant properties. ASA may act as an antioxidant, inhibiting chromosomal damage induced by the free radicals generated by doxorubicin [9]. Also, Hsu and Li [10] demonstrated that the inhibition of oxidative stress by ASA is concentration-dependent *in vitro*, and it was proposed that the antioxidant activity of ASA might furnish cancer chemoprotection in humans.

Abbreviations used: ASA, acetylsalicylic acid; FBS, fetal bovine serum; FU, fluorescence units; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H_2DCF-DA, 2′,7′-dichlorofluorescin diacetate; MDR, multidrug resistance; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide; NSAID, non-steroidal anti-inflammatory drug; PBMC, peripheral blood mononuclear cell; PG, prostaglandin; Pgp, P-glycoprotein; PI, propidium iodide; ROS, reactive oxygen species; RT–PCR, reverse transcriptase–PCR; VCR, vincristine.

[1]To whom correspondence should be addressed (email gilma.trindade@gmail.com).

Cell death is an essential phenomenon in normal development and homoeostasis, but it also plays a crucial role in various pathologies. Apoptosis is genetically regulated and provides a vital protective mechanism against the development of neoplasms by removing cells with DNA damage. Thus, inhibition of apoptosis confers a survival advantage to cells harbouring genetic alterations and might promote the acquisition of further mutations that cause neoplastic progression and also contribute to the development of resistance to chemotherapy [11]. The *bcl-2* gene is the first member of a gene family that regulates apoptosis [12]. Overexpression of *bcl-2* gene increases tumoral cell survival, protecting them from chemotherapic toxicity [13] and preventing apoptosis. In contrast, apoptosis induction is one of the central actions by which P53 protein promotes tumour suppression [14]. The P53 protein, wild-type, is a nuclear phosphoprotein that acts as a transcriptional factor for cellular growth genes, signalling DNA repair when it suffers damage or inducing apoptosis when the damage is too extensive for repair [15].

One of the major causes of chemotherapeutic failures in cancer treatment is the development of different types of resistance. MDR (multidrug resistance) is a phenomenon by which tumours, which initially respond to a determined chemotherapy, acquire resistance to chemically and non-chemically related drugs. The best-understood mechanism of MDR is the one conferred by the membrane Pgp (P-glycoprotein or ABCB1), which acts by pumping several unrelated drugs out of treated cells [16]. Despite the multifactorial nature of the resistance process, MDR phenotypes exhibit some common characteristics: (i) resistance to non-related drugs [17,18], (ii) augmented expression of Pgp or related proteins [16], (iii) the ability to extrude rhodamine 123 dye [19] and (iv) the reversion of their resistance induced by agents such as trifluoperazine, verapamil and cyclosporin A [20,21]. So, with the intention of creating a biological model that permits comparative studies between MDR tumoral cell lines and non-MDR tumoral cells, Rumjanek et al. [22] established an *in vitro* MDR model utilizing VCR (vincristine) to induce a resistant erythroleukaemic cell line. These MDR cells were named K562-Lucena1 (Lucena) to distinguish this line from its K562 parental cell line. Lucena cells exhibit the characteristics of MDR cells cited above [23–26].

Thus the aim of the present study was to analyse whether the effect of ASA differs between non-MDR and MDR cells with regard to cytotoxicity, induction of apoptosis and/or necrosis, production of oxidative effects and the capacity to alter the expression of certain genes. In addition, we evaluated the response of normal lymphocytes to different treatments with ASA.

MATERIALS AND METHODS

Cell line and culture conditions

The K562 and Lucena are human erythroleukaemic cell lines. They were obtained from the Tumoral Immunology Laboratory at the Medical Biochemistry Institute of the Federal University of Rio de Janeiro, Rio de Janeiro, Brazil. The cells were grown at 37°C in disposable plastic flasks containing RPMI 1640 (Gibco) medium supplemented with sodium bicarbonate (0.2 g/l) (Vetec), L-glutamine (0.3 g/l) (Vetec), Hepes (25 mM) (Acros), 2-mercaptoethanol (5×10^{-5} M) (Sigma), FBS (fetal bovine serum; 10%; Gibco), 1% of antibiotic [penicillin (100 units/ml) and streptomycin (100 mg/ml) Gibco] and antimycotic (0.25 mg/ml; Sigma). Lucena cells were grown under the same conditions as K562 cells, with the addition of 60 nM VCR (Sigma) in the culture medium.

Treatment of cells: ASA exposure

ASA (Vetec) was purified by recrystallization [27] and stored in a vacuum dessicator. Stock solutions of ASA (1.0 M) dissolved in 100% ethanol (Vetec) were freshly prepared for each experiment with the pH adjusted to 7.4. They were mixed with the culture medium free of 2-mercaptoethanol to achieve concentrations of 2.5, 5, 10 and 15 mM. For all the assays, untreated cells (control cells) received only absolute ethanol to achieve the maximum concentration of ethanol of the treated cells that did not alter its cellular viability (Supplementary Figure S1 at http://www.bioscirep.org/bsr/031/bsr0310391add.htm).

Cell viability assay

The viability of K562 and Lucena cells was assessed by Trypan Blue exclusion immediately, 24, 48 and 72 h after incubation with ASA. This assay was used to distinguish between proliferation inhibition and cytotoxicity. Cells were grown for 2 days (K562) and for 3 days (Lucena) before the experiments were performed [28]. Cells were then centrifuged, washed twice with PBS (Ca^{+2}–Mg^{+2}-free) and suspended in RPMI 1640 medium without 2-mercaptoethanol to 5×10^5 cells/ml. VCR was removed from the medium before the experiments. The cells were treated with different concentrations of ASA (2.5, 5, 10 or 15 mM) or without ASA (control cells) in 24-well culture plates. Each experiment was performed three times using triplicates in each experiment. The concentration of 15 mM was not employed in later tests because it enhanced cytotoxicity (see Results section).

Detection of apoptosis/necrosis by Annexin V/PI (propidium iodide) staining

Quantitative determination of apoptotic and/or necrotic cells was performed after incubation with 2.5, 5 and 10 mM of ASA for 48 h through a reaction with Annexin V–FITC and PI. Cells were washed twice with PBS (2×10^5 cells/well), suspended in 250 μl of binding buffer diluted 10 × [0.1 M Hepes/NaOH (pH 7.4), 1.4 M NaCl, 25 mM $CaCl_2$] plus 20 μl of Annexin V–FITC solution diluted in binding buffer (1:10). After 20 min incubation in the dark, PI was added (5 μl) and the acquisition by cells was detected by means of a flow cytometer (FACSCalibur, BD Biosciences). The percentages of total cells that underwent apoptosis/necrosis were calculated with the Cell Quest Pro program. Annexin V–$FITC^+$/PI^- cells were counted as early

apoptosis; Annexin V–FITC$^+$/PI$^+$ and Annexin V–FITC$^-$/PI$^+$ cells were counted as necrosis [29].

Sensitivity of normal lymphocytes to ASA

The normal lymphocytes were obtained by the separation of heparinized blood from healthy male volunteers by Ficoll-Histopaque (Sigma) density gradient centrifugation. After washing twice with PBS, the fraction of lymphocytes was suspended in RPMI 1640 medium with 5% FBS. The suspension (1×10^6 cells/well) was incubated in culture plates, stimulated with lyophilized PHA (phytohaemagglutinin) (2%) and incubated for 24 h at 37°C. After 24 h, the cells were treated with 2.5, 5 and 10 mM of ASA. The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide] assay was used to monitor cell proliferation immediately, 24, 48 and 72 h after incubation with ASA, according to protocol [28]. Briefly, lymphocytes, after incubation, were washed with PBS and 200 μl of RPMI 1640 medium free of 2-mercaptoethanol and 20 μl of MTT (5 mg/ml) were added to each well. The plates were incubated for 4 h at 37°C. The medium was removed and formazan crystals were dissolved in 200 μl of DMSO (Sigma) with gentle shaking. The absorbance values at 490 nm were determined on a multiwell plate reader (ELX 800 Universal Microplate Reader; Bio-TEK). The MTT assay was utilized because of the difficulty in the separation of lymphocytes for counting by Trypan Blue exclusion.

Assessment of intracellular ROS (reactive oxygen species) formation

Suspensions of both cell lines (5×10^5 cell/ml) (control cells and cells treated with 2.5, 5 and 10 mM of ASA during 24 and 48 h) were washed twice with PBS and incubated for 30 min at 37°C with the fluorigenic compound H_2DCF-DA (2′,7′-dichlorofluorescin diacetate; 40 μM; Molecular Probes) [30]. H_2DCF-DA passively diffuses through cellular membranes and, once inside, the acetate is cleaved by intracellular esterases. Thereafter, the non-fluorescent compound H_2DCF is oxidized by ROS into a fluorescent compound [DCF (2′,7′-dichlorofluorescein)]. Once loaded with H_2DCF-DA, the cells were washed twice with PBS and resuspended in fresh PBS. Each treatment was performed in triplicate. Aliquots from 160 μl of each sample (three replicates) were placed into an ELISA plate and the fluorescence intensity determined during 90 min at 37°C, using a fluorimeter (Victor 2, PerkinElmer) with λ_{ex} and λ_{em} of 485 and 520 nm respectively. ROS levels were expressed in terms of fluorescence area and were obtained by integrating the FU (fluorescence units) over the measurement time (90 min) and expressed as FU min.

Evaluation of the gene expression by RT–PCR (reverse transcriptase–PCR)

The mRNA expression of *bcl-2*, *cox-2* and *p53* was evaluated by RT–PCR. After the incubation with different concentrations of ASA (2.5, 5 and 10 mM) for 48 h, total RNA from K562 and Lucena cells (1×10^6 cell/ml) was extracted using TRIzol® reagent (Invitrogen) according to the protocol suggested by the manufacturer. The RNA extracts were qualitatively evaluated by electrophoresis in 1% agarose gel, and quantified with the Qubit™ Fluorometer (Invitrogen). The Quant-iT™ RNA Assay Kit (Invitrogen) was used, and calibration was performed using a two-point standard curve. The relationship between the two standards and a curve-fitting algorithm was used to calculate the concentrations of the RNA samples. Total RNA from each pool was used as template for the RT–PCR with the AP primer [5′-GGCCACGCGTCGACTAGTAC(T)17-3′; Invitrogen]. The cDNA synthesis was carried out using the enzyme RT SuperScript III (Invitrogen) according to the protocol suggested by the manufacturer. The cDNA obtained was used as template for the gene amplification. Specific primers were used for *bcl-2* (bcl-2-F: 5′-GACTTCGCCGAGATGTCCAG-3′; bcl-2-R: 5′-CAGGTGCCGGTTCAGGTACT-3′, giving an expected PCR product of 225 bp), *cox-2* (cox-2-F: 5′-TGAAACCCACTCCAAACACAG-3′; cox-2-R: 5′-TCATCAGGCACAGGAGGAAG-3′, giving an expected PCR product of 232 bp), *p53* (p53-F: 5′-CTGAGGTTGGCTCTGACTGTACCACCATCC-3′; p53-R: 5′-CTCATTCAGCTCTCGGAACATCTCGAAGCG-3′, giving an expected PCR product of 370 bp), GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (GAPDH-F: 5′-ATGGCACCGTCAAGGCTGAG-3′; GAPDH-R: 5′-GCAGTGATGGCATGGACTGT-3′, giving an expected PCR product of 379 bp) gene expression. PCR was carried out in a 12.5 μl reaction volume containing 1.25 μl of 10 × PCR buffer, 0.25 μM of each primer, 0.25 mM of each dNTP, 0.375 mM of $MgCl_2$, 0.1 unit of Platinum Taq DNA polymerase (Invitrogen) and 0.5 μl of cDNA solution. The reaction mixture was incubated at 94°C for 2 min followed by 25 [GAPDH (Lucena)], 28 [GAPDH (K562)], 35 (*p53*), 38 [*bcl-2* (Lucena) and *cox-2*] and 40 [*bcl-2* (K562)] cycles {denaturation at 94°C for 30 s [GAPDH, *bcl-2* (Lucena) and *p53*], 15 s [*bcl-2* (K562) and *cox-2*], annealing at 56°C for 30 s (GAPDH), 63°C [*bcl-2* (Lucena) and *p53*], 65°C [*bcl-2* (K562)], 55°C for 45 s [*cox-2* (K562)] and 65°C [*cox-2* (Lucena)] and extension at 72°C for 30 s} and with an additional (final) extension at 72°C for 10 min. The PCR products were separated by electrophoresis on an 1.5% (w/v) agarose gel and stained with Sybr Safe™ (Invitrogen) for densitometric analysis. Calculation of absorbance was performed with the ONE-Dscan software (Scanalytics) for each gene and was normalized to corresponding GAPDH values.

Analysis of human bcl-2, cox-2 and p53 promoters

To search for transcription factor binding sites, sequences containing 2000 bp of *bcl-2*, *cox-2* and *p53* proximal promoters were identified at GenBank®. For human *bcl-2*, *cox-2* and *p53*, the sequences used were CCDS11981, CCDS1371 and CCDS11118 respectively. The potential transcription factor binding sites were localized using the MatInspector program [31], considering only core sequences with 100% identity.

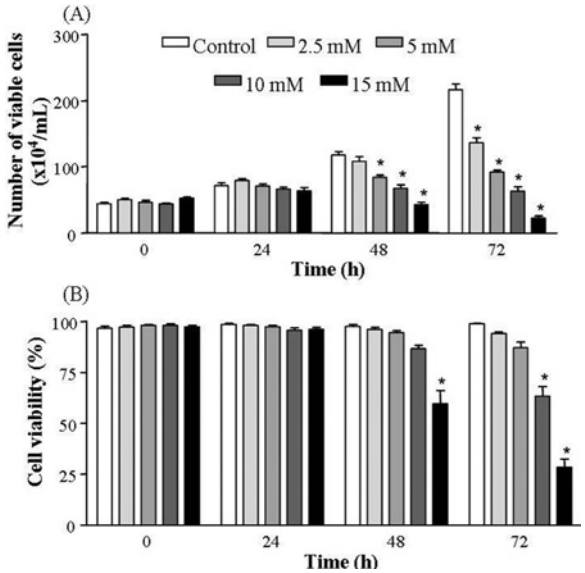

Figure 1 Cell viability of K562 cell line exposd to ASA
(**A**) Number of viable cells ($\times 10^4$) and (**B**) cell viability (%) of K562 cells treated with different concentrations of ASA at different times of exposure, according to Trypan Blue exclusion test. Results are expressed as means $\pm$ S.E.M. $^*P < 0.05$ when compared with control.

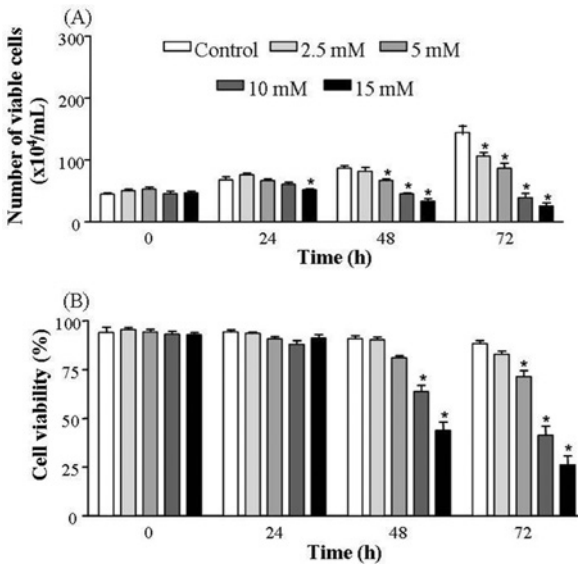

Figure 2 Cell viability of Lucena cell line expose at ASA
(**A**) Number of viable cells ($\times 10^4$) and (**B**) cell viability (%) of Lucena cells treated with different concentrations of ASA at different times of exposure, according to Trypan Blue exclusion test. Results are expressed as means $\pm$ S.E. $^*P < 0.05$ when compared with control.

Statistical analysis

At least three independent experiments were carried out using triplicates in each experiment. Results were expressed as means ($\pm$ S.E.M.) and analysed by ANOVA followed by the Tukey multiple range test. ANOVA assumptions (normality and homogeneity of variances) were previously verified. Significance level was fixed at $P < 0.05$.

RESULTS

Cell viability

Viability was significantly different in K562 and Lucena cells treated with different concentrations of ASA (2.5, 5, 10 and 15 mM) when compared with the control group ($P < 0.05$). The effect of ASA treatment was concentration- and time-dependent, in both cell lines (Figures 1A, 1B, 2A and 2B). The 2.5 mM concentration inhibited cellular proliferation ($P < 0.05$) only at 72 h but did not show toxic effects in either cell line. The 5 mM concentration inhibited cellular proliferation from 48 h in both cell lines, except in Lucena cells at 72 h, where this concentration was cytotoxic. The 10 mM ASA concentration inhibited the proliferation of K562 cells at 48 h. This concentration was cytotoxic to Lucena cells at 48 h and to K562 cells at 72 h. The 15 mM concentration in Lucena cells inhibited proliferation as soon as 24 h. From 48 h on, this concentration was toxic to both cell lines.

Detection of apoptosis/necrosis by annexin-V/PI staining

As shown in Figure 3, K562 and Lucena control cells, presented low staining with annexin V and PI. The same occurred with 2.5 and 5 mM of ASA treatment. However, after incubation with 10 mM ASA, K562 cells underwent early apoptosis (stained positive for annexin V) as well as necrosis (stained positive for PI and positive for annexin V plus PI) (Figure 3A). However, the Lucena cells only showed a statistically significant difference ($P < 0.05$) in staining to annexin V plus PI and only PI when treated with 10 mM, indicating death by necrosis (Figure 3B).

ASA effects in normal PBMCs (peripheral blood mononuclear cells)

The ASA effects in normal lymphocytes are presented in Figure 4. ASA induced cytotoxicity only in cells treated with 10 mM at 72 h when compared with the control cells ($P < 0.05$). At this time point, proliferation was inhibited with 5 mM when compared with its respective control ($P < 0.05$).

Antioxidant effects of ASA

K562 cells treated with 10 mM of ASA at 24 h showed a significant decrease ($P < 0.05$) in the amount of ROS. This was also observed in Lucena cells treated with 5 and 10 mM (Figure 5A). After 48 h of incubation, K562 cells treated with 5 and 10 mM of ASA showed a significant decrease ($P < 0.05$) in the amount

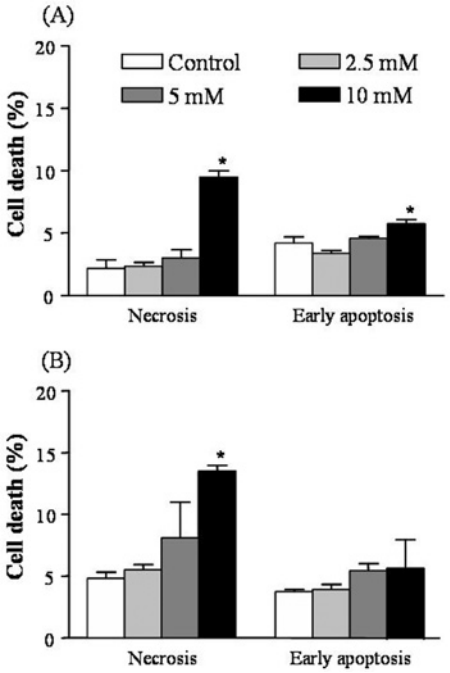

Figure 3 ASA induces cellular death
Induction of cell death (%) by early apoptosis and/or necrosis in (**A**) K562 and (**B**) Lucena cells treated with 2.5, 5 and 10 mM ASA for 48 h. Results are expressed as means ± S.E.M. $*P < 0.05$ when compared with control.

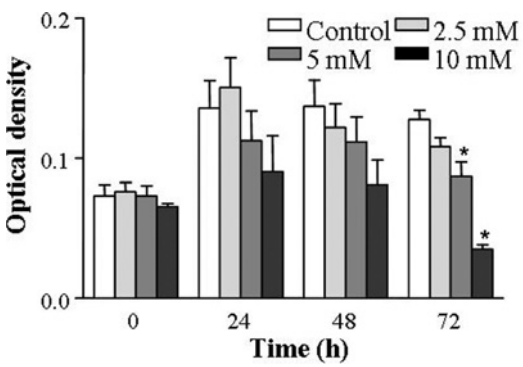

Figure 4 ASA did not alter sensitivity in normal PBMCs
Sensitivity of lymphocytes treated with 2.5, 5 and 10 mM of ASA at different times, as measured by MTT assay. Results are expressed as means ± S.E.M. $*P < 0.05$ when compared with control.

of ROS, while in Lucena cells this decrease ensued only with 10 mM of ASA (Figure 5B).

Evaluation of the gene expression by RT–PCR

The ASA treatment did not affect the expression of the *bcl-2* gene (Figure 6A), although it induced apoptosis and/or necrosis in K562 and Lucena cells. Additionally, there was no difference

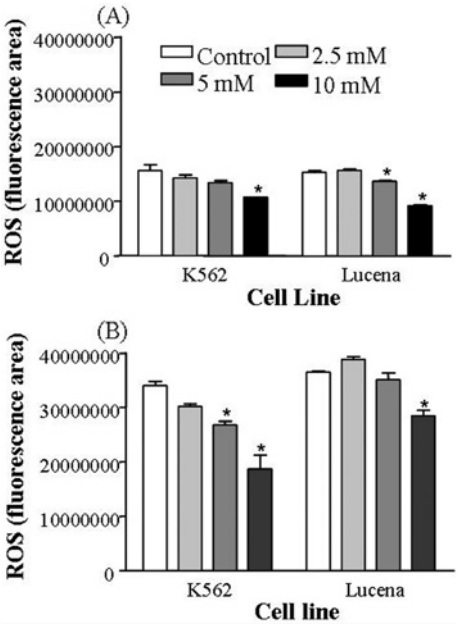

Figure 5 Antioxidant effects of ASA
ROS production (fluorescence area) in K562 and Lucena cell lines treated with 2.5, 5 and 10 mM ASA at (**A**) 24 and (**B**) 48 h. Results are expressed as means ± S.E.M. $*P < 0.05$ when compared with control.

in basal levels between these cell lines ($P > 0.05$). The basal *p53* expression in Lucena cells was higher than in K562 cells (Figure 6B). The same pattern was observed for *cox-2* expression (Figure 6C). K562 cells showed an increase in *p53* and *cox-2* expression which was concentration-dependent. In contrast, *p53* expression in Lucena cells was increased at 2.5 mM and decreased at 10 mM ASA. An increase in *cox-2* expression was observed just in 2.5 and 5 mM of ASA.

DISCUSSION

ASA is capable of preventing and decreasing some types of cancer, including chronic lymphocytic leukaemia and acute myeloid leukaemia [32]. However, the mechanism of action of ASA still requires study to validate its possible antitumoral and anti-MDR properties. One of the major challenges in cancer treatment is to find a drug with antitumoral action in non-MDR and MDR cells. ASA showed this ability by inhibiting the cellular proliferation or inducing toxicity in K562 and Lucena cells [22,33]. Moreover, at some concentrations and time-points, the MDR cells were more sensitive than their parental line K562. Also, Trindade et al. [6] demonstrated similar results when these cells were exposed to photodynamic therapy with Methylene Blue and visible light. Indomethacin, another NSAID, also inhibits proliferation in K562 cells [34].

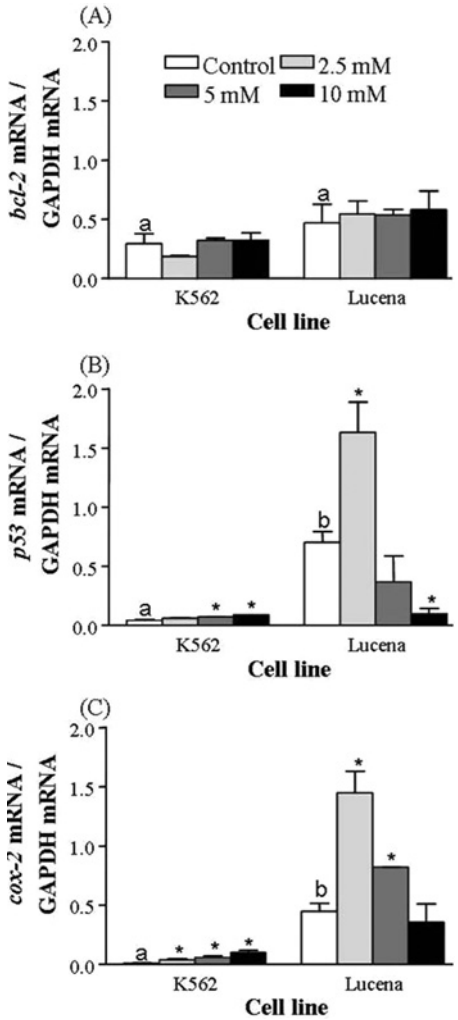

Figure 6 Effect of ASA on gene expression
Analysis of gene expression in K562 and Lucena cells treated with 2.5, 5 and 10 mM ASA at 48 h. (**A**) *bcl-2* mRNA expression. (**B**) *p53* mRNA expression. (**C**) *cox-2* mRNA expression. Results are expressed as means $\pm$ S.E.M. $*P < 0.05$ when compared with control. Similar letters indicate the absence of significant differences between controls ($P > 0.05$).

In the last few years, *in vitro* studies have shown that the cytotoxic effects of several drugs in human tumoral cell lines are mediated by apoptosis [35]. In the present work, K562 and Lucena cells died from apoptosis and/or necrosis only when treated with a concentration of 10 mM for 48 h. This result suggests that K562 and Lucena cells are less sensitive to ASA treatment when compared with the lines studied by Bellosillo et al. [32]. Klampfer et al. [36] attributed the reduced sensitivity of K562 cells to sodium salicylate-induced apoptosis to the presence of the *t*(9;22) chromosomal translocation (Philadelphia chromosome) and the expression of the Bcr–Abl fusion protein in this cell line. The ultimate effect of the Bcr–Abl product seems to be an enhancement of cell survival [37]. However, at 10 mM ASA, a secondary post-apoptotic necrosis (delayed apoptosis) was observed. There is evidence that Pgp, when overexpressed in MDR cells, induces resistance to programmed cell death [38]. A compatible result, with Lucena cells, was obtained in the present work.

The comparison between tumoral cells and normal lymphocytes indicates that the latter are more resistant to ASA at all concentrations tested. Bellosillo et al. [32] also observed that normal lymphocytes are more resistant to ASA than chronic lymphocytic leukaemia cells (B-CLL), with doses up to 7.5 mM.

The toxicity of ASA in K562 and Lucena cells is not due to the generation of ROS since ASA showed antioxidant activity in both cell lines. This result is expected, considering that PG induces ROS generation [39] and that ASA inhibits Cox-2 action, reversing this process. Antunes et al. [9] found a inhibition in the total number of chromosomal aberrations and aberrant metaphases caused by ROS released by doxorubicin in lymphocytes incubated for 24 h with ASA (25, 50 or 100 μg/ml), which supports its role as an antioxidant agent.

Considering gene expression, in the present study, the basal expression of *bcl-2* gene was similar in the controls of both cell lines. These results are in agreement with those of Wagner-Souza et al. [40] who compared Bcl-2 expression in K562 and Lucena cell lines and demonstrated that there is no difference between them. Also, treatment with ASA for 48 h demonstrated that the transcriptional expression of *bcl-2* also was not altered, since it is similar to control cells in K562 and Lucena cells. It can be suggested that the mechanism of cell death induced by ASA is independent of *bcl-2*. Also analysing Bcl-2 protein expression, Klampfer et al. [36], demonstrated that treatment with sodium salicylate after 5 and 18 h did not alter the expression of the Bcl-2 proteins in the TF-1 acute myeloid leukaemia cell line. However, several authors reported that Bcl-2 protein expression in some tumoral cells is enhanced by chemotherapeutic drugs [41]. In relation to the biological model used in the present work, Zhang et al. [34] observed that indomethacin (NSAID) down-regulates *bcl-2* gene expression in K562 cells.

Several lines of evidence indicate that *p53* transcriptional activity does not always correlate with its apoptotic activity [42]. In the present work, a difference in the profile of *p53* expression between K562 and Lucena cells was observed: the first was concentration-dependent and the second was bell-shaped. The basal levels of *p53* mRNA were significantly different between K562 and Lucena cells and in K562 control cells, *p53* mRNA levels were very low. According to Cavalcanti Júnior et al. [43] a mutation of the *p53* gene (stop codon) in the K562 cell line is responsible for the absence of wild-type P53 protein expression. On the other hand, although the wild-type P53 protein is not present in the Lucena cells [43], its basal expression of mRNA in the present study was significantly higher. Several studies have shown increased levels of P53 in tumour cells after treatment with a variety of DNA-damaging agents [44]. This is generally observed in wild-type P53; however, mutant P53 has been shown to possess its own activities, often not present in the original wild-type P53 protein, which can actively contribute to various aspects of tumour progression. Such activities are commonly described as mutant P53 gain-of-function [45]. Furthermore, the mutant P53 has been described to induce transcription of the promoter of the *mdr1* gene, which expresses the Pgp protein, while wild-type

P53 does not induce transcription of this promoter [46]. Other studies have demonstrated that nuclear P53 accumulation is often associated with Pgp expression in primary cancer, and simultaneous expression of P53 and Pgp is associated with a series of molecular events resulting in a more aggressive phenotype, drug resistance and poor prognosis [47]. Considering the premises above, a *p53* basal expression linked with Pgp expression reinforces the MDR phenotype in the Lucena cell line. In this sense, the down-regulation of *p53* observed in Lucena cells in the higher ASA concentrations may explain the death of these cells.

Several mechanisms of growth inhibition by NSAIDs in cancer cell lines have been proposed. There is particular interest in the inhibition of Cox-2 expression because it is associated with tumour progression [48] and NSAIDs, such as ASA, are known to inhibit cyclo-oxygenase enzymes [49]. It is relevant to emphasize that *p53* and *cox-2* genes express similarity, considering basal as well as treated cells' expression. This similarity suggests an interaction between *cox-2* and *p53* expression. In the present work, ASA induced the expression of *cox-2* in K562 and Lucena cells. The increase of the *cox-2* expression may indicate that ASA actually inhibited the Cox-2 protein. However, Jin et al. [50] demonstrated that the non-Cox-2-inhibition by another drug [(*R*)-flurbiprofen] was most effective at reducing proliferation of gastric cancer cells *in vitro*. (*R*)-Flurbiprofen prevented the metastatic characteristics of gastric cancer cells *in vitro*, and reduced tumour size and metastasis *in vitro*, when gastric cancer cells were injected into nude mice. (*R*)-Flurbiprofen also affected MDR, increasing the sensitivity of resistant gastric cancer cells to chemotherapeutic agents. The controversial results suggest that different tumoral cell lines can present distinct responses to NSAIDs.

The results of this work show that ASA possesses an antioxidant activity, the capacity to alter *p53* and *cox-2* gene expression and cytotoxic activity to both cell lines. Taken together, these results demonstrate a possible anti-MDR role in addition to the possible antitumoral property demonstrated by the lower sensitivity of normal lymphocytes to ASA treatment. These findings corroborate the literature suggesting the importance of further studies to investigate the potential of NSAIDs as clinical treatment of cancer.

AUTHOR CONTRIBUTION

Michele Carrett-Dias performed all of the experiments, discussed the results and prepared the paper. Ana Paula de Souza Votto co-advised the experiments involving cell culture, participated in the discussion of results and review of the paper. Daza de Moraes Vaz Batista Filgueira collaborated in the discussion of results and revisions of the paper. Daniela Volcan Almeida collaborated on the experiments involving malecular biology. Adriana Lima Vallochi advised on flow cytometry experiments. Marcelo Gonçalves Montes D'Oca advised in the process of purification of the ASA Luis Fernando Marins advised the experiments involving molecular biology, and participated in the discussion of results and review of the paper. Gilma Santos Trindade advised all stages of the research.

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online data

Original Paper

SUPPLEMENTARY ONLINE DATA

Anti-MDR and antitumoral action of acetylsalicylic acid on leukaemic cells

Michele CARRETT-DIAS*, Ana Paula de Souza VOTTO*, Daza de Moraes Vaz Batista FILGUEIRA*, Daniela Volcan ALMEIDA*, Adriana Lima VALLOCHI†, Marcelo Gonçalves Montes D'OCA‡, Luis Fernando MARINS*§ and Gilma Santos TRINDADE*§[1]

*Programa de Pós-graduação em Ciências Fisiológicas – Fisiologia Animal Comparada, Universidade Federal do Rio Grande (FURG), 96201-900 Rio Grande, RS, Brazil, †Fundação Oswaldo Cruz, Instituto Oswaldo Cruz, 21045-900 Rio de Janeiro, RJ, Brazil, ‡Escola de Química e Alimentos, FURG, 96201-900 Rio Grande, RS, Brazil, and §Instituto de Ciências Biológicas, FURG, 96201-900 Rio Grande, RS, Brazil

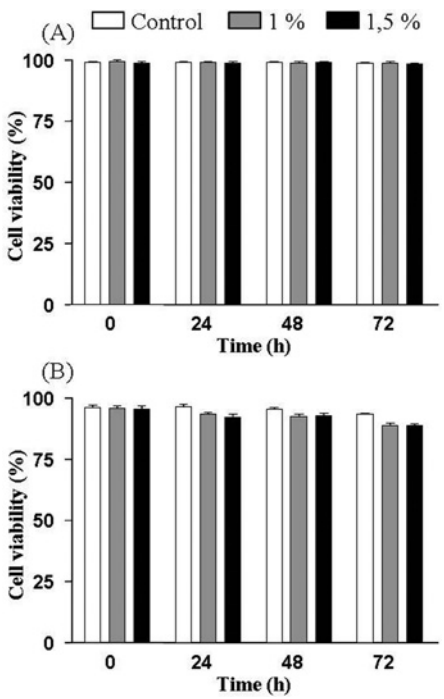

Figure S1 Cell viability of K562 and Lucena cell line exposed to ethanol vehicle

Cell viability (%) of K562 cells (**A**) and Lucena cells (**B**) exposed to different concentrations of ethanol at different times of exposure, according to Trypan Blue exclusion test. Results are expressed as means ± S.E.M. $*P < 0.05$ when compared with control.

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[1]To whom correspondence should be addressed (email gilma.trindade@gmail.com).