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Quercetin decreases intracellular GSH content and potentiates the apoptotic action of the antileukemic drug arsenic trioxide in human leukemia cell lines

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ABSTRACT

Arsenic trioxide (ATO) is an effective therapeutic agent for the treatment of acute promyelocytic leukemia, but successful application of this agent may occasionally require the use of sensitizing strategies. The present work demonstrates that the flavonoids quercetin and chrysin cooperate with ATO to induce apoptosis in U937 promonocytes and other human leukemia cell lines (THP-1, HL-60). Co-treatment with ATO plus quercetin caused mitochondrial transmembrane potential dissipation, stimulated the mitochondrial apoptotic pathway, as indicated by cytochrome c and Omi/Htra2 release, XIAP and Bcl-X_L down-regulation, and Bax activation, and caused caspase-8/Bid activation. Bcl-2 over-expression abrogated cytochrome c release and apoptosis, and also blocked caspase-8 activation. Quercetin and chrysin, alone or with ATO, decreased Akt phosphorylation as well as intracellular GSH content. GSH depletion was regulated at the level of L-buthionine-(S,R)-sulfoximine (BSO)-sensitive enzyme activity, and N-acetyl-L-cysteine failed both to restore GSH content and to prevent apoptosis. Treatment with BSO caused GSH depletion and potentiated ATO-provoked apoptosis, but did not affect apoptosis induction by ara-C and cisplatin. As an exception, ATO plus quercetin failed to elicit Akt de-phosphorylation and GSH depletion in NB4 acute promyelocytic leukemia cells, and correspondingly exhibited low cooperative effect in inducing apoptosis in this cell line. It is concluded that GSH depletion explains at least in part the selective potentiation of ATO toxicity by quercetin, and that this flavonoid might be used to increase the clinical efficacy of the antileukemic drug.

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Abbreviations: APL, acute promyelocytic leukemia; Ara-C, 1-β-D-Arabinofuranosylcytosine; ATO, arsenic trioxide; BSO, L-Buthionine-(S,R)-sulfoximine; Cisplatin, CDDP, cis-platinum(II)-diammine dichloride; DAPI, 4,6-diamino-2-phenylindole; FITC, fluorescein isothiocyanate; γ-GCS, gamma-glutamylcysteine sinthetase, glutamate-cysteine ligase; GSH-OEt, reduced glutathione ethyl ester; HSP, heat-shock protein; JNK, Jun N-terminal kinase; mAb, monoclonal antibody; MAPK, mitogen-activated protein kinase; pAb, polyclonal antibody; MEK/ERK, mitogen-induced extracellular kinase/extracellular signal-regulated kinase; NAC, N-acetyl-L-cysteine; PI3K, phosphatidylinositol 3-kinase; PI, propidium iodide; ROS, reactive oxygen species; z-IETD-fmk, Z-Ile-Glu(OMe)-Thr-Asp(OMe)-CH_2F; z-VAD-fmk, Z-Val-Ala-Asp(OMe)-CH_2F.

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1. Introduction

Arsenic trioxide (ATO, Trisenox™) is an effective therapeutic agent for the treatment of relapsed and refractory acute promyelocytic leukemia (APL) [1]. The administration of low, physiologically tolerable concentrations of ATO (0.25–2 μM in plasma) causes APL cytoreduction by inducing terminal differentiation and/or apoptosis. Although this response was originally explained by the capacity of the drug to disrupt the PML-RARα fusion protein characteristic of most APLs, ATO causes other biochemical effects which are also important for apoptosis, including direct binding to the adenosine nucleotide translocator (ANT) and induction of mitochondrial pore permeabilization [2], as well as inhibition of mitochondrial respiration and generation of reactive oxygen species (ROS) [3]. Because of this, ATO also causes apoptosis in tumour cells types other than APL, a response which opens the possibility of extending the therapeutic applications of the drug [4]. Nevertheless, the relatively low sensitivity of most tumour cell types to ATO may require the generation of sensitizing strategies or appropriate drug combinations, to increase the ATO efficacy and reduce its dosage to clinically achievable concentrations.

Flavonoids are a class of phenolic compounds widely distributed in the plant kingdom. These agents display diverse biological activities, including prevention of cancer initiation and inhibition of tumour progression [5]. In this regard, quercetin was demonstrated to cause death by apoptosis in different cancer cell lines [6–9], and its potential efficacy as antitumour agent was corroborated in mouse models and phase I clinical trials [10,11]. Moreover, quercetin was occasionally reported to potentiate apoptosis induction and/or cell growth inhibition by typical antitumour drugs such as cisplatin and cytosine arabinoside (ara-C) [12–15]. At the biochemical level, quercetin and other flavonoids directly interact with mitochondria causing respiratory chain inhibition [16]; de-regulates cell signalling pathways, causing MAPK activation or inhibition [17,18] and PI3K/Akt inhibition [8,14]; and may also affect the redox state by altering the intracellular glutathione content [19,20]. This property is particularly important for the present purpose, since the glutathione (GSH)-based redox system is a strict determinant of ATO sensitivity. Thus, the apoptotic action of ATO inversely correlates with the endogenous level of GSH or GSH-associated enzymes in different leukemia cell types [21–23], and treatments that experimentally deplete or enhance the GSH content exacerbate or decrease, respectively, ATO toxicity [21–24]. In addition, we recently reported that down-regulating the PI3K/Akt, MEK/ERK and JNK pathways decreased intracellular GSH and increased ATO toxicity in leukemia cells [25,26].

With these considerations in mind, we asked whether co-treatment with low toxic quercetin concentrations could represent a useful manner of improving the apoptotic action of ATO. The obtained results indicate that quercetin cooperates with ATO in inducing apoptosis in human myeloid leukemia cells, although the efficacy depends very much on the used cell line. The potentiation of ATO toxicity by quercetin correlates and may be explained at least in part by the capacity the flavonoid to down-regulate Akt phosphorylation and decrease the intracellular GSH content in the leukemia cell model.

2. Materials and methods

2.1. Chemicals

All components for cell culture were obtained from Invitrogen, Inc. (Carlsbad, CA). Monochlorobimane and rhodamine 123 were obtained from Molecular Probes (Eugene, OR). 4,6-Diamino-2-phenylindole (DAPI) was obtained from Serva (Heidelberg, Germany). The kinase inhibitors U0126, SB203580, SP600125, and LY294002, and the caspase inhibitors benzyloxy-carbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk, non-specific) and Z-Ile-Glu(OMe)-Thr-Asp(OMe)-CH_2F (z-IETD-fmk, specific for caspase-8), were obtained from Calbiochem (Darmstad, Germany). Rabbit polyclonal antibodies (pAbs) against human Akt, phospho-Akt (Ser^{473}), p44/42 MAPK, phospho-p44/42 MAPK (Thr^{202}/Tyr^{204}), p38 MAPK, phospho-p38 MAPK (Thr^{180}/Tyr^{182}), SAPK/JNK and phospho SAPK/JNK (Thr^{183}/Tyr^{185}), were obtained from Cell Signaling Technology (Beverly, MA). Mouse anti-pigeon cytochrome *c* monoclonal antibody (mAb) clone 7H8.2C12, mouse anti-Bax mAb clone 6A7, and rabbit anti-rat Bcl-x pAb, were obtained from BD PharMingen (San Diego, CA). Mouse anti-human Bcl-2 (100) mAb, rabbit anti-human Bax (N-20) pAb, and goat anti-human Bid (C-20) pAb, were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse anti-XIAP (MIHA/ILP-a) mAb was obtained from MBL International Corporation (Woburn, MA, USA). Mouse anti-human 70-kDa heat-shock protein (HSP70) mAb (clone C92F3A-5, which specifically recognizes the stress-inducible form of HSP70). All peroxidase and fluorescein isothiocyanate (FITC)-conjugated IgG antibodies were obtained from DAKO Diagnósticos, S.A. (Barcelona, Spain). All other reagents were from Sigma (Madrid, Spain).

2.2. Cells and treatments

The human leukemia cell lines U937 and THP-1 (promonocytic), HL-60 (myelomonocytic), and NB4 (acute promyelocytic), and Bcl-2-transfected U937 cells (U4 clone, kindly provided by Dr. Jacqueline Bréard, INSERM 461, Chatenay Malabry, France) [27] were routinely grown in RPMI 1640 supplemented with 10% (v/v) heat-inactivated calf serum, 0.2% sodium bicarbonate and antibiotics in a humidified 5% CO_2 atmosphere at 37 °C. For experiments, 16–24 h before the initiation of the treatments the cell concentration was adjusted at approximately 10^5 cells/ml. In some experiments aimed at analyzing Akt activation, the cells were incubated for 16 h with low (1.5%, v/v) serum concentration to decrease the basal level of phosphorylated Akt, prior to stimulation with insulin. Stock solutions of quercetin and chrysin (100 mM), U0126 (2.63 mM), SB203580, SP600125 and LY294002 (20 mM), z-VAD-fmk and z-IETD-fmk (25 mM), monochlorobimane (200 mM), and N-acetyl-L-cysteine (NAC, 3 M), were prepared in dimethyl sulfoxide; stock solutions of butylated hydroxyanisole (500 mM) and Trolox (100 mM) were prepared in absolute ethanol; and stock solutions of cisplatin (3.3 mM) and Tiron (10 mM) were prepared in distilled water. All these solutions

were stored at −20 °C. Stock solutions of DAPI (10 (g/ml), PI and rhodamine 123 (1 mg/ml) were prepared in phosphate-buffered saline (PBS); and a stock solution of ATO (100 mM) was prepared in distilled water. These solutions were stored at 4 °C. Stock solutions of ara-C (10 mM in RPMI), L-buthionine-(S,R)-sulfoximine (BSO, 50 mM in distilled water), and reduced glutathione ethyl ester (GSH-OEt, 50 mM in PBS), were prepared just before application.

2.3. Flow cytometry

The analysis of samples was carried out using an EPICS XL flow cytometer (Coulter, Hialeah, FL) equipped with an air-cooled argon laser tuned to 488 nm. The specific fluorescence signals corresponding to FITC and rhodamine 123 were collected with a 525-nm band pass filter, and the signal corresponding to PI with a 620-nm band pass filter.

2.4. Determination of apoptosis

Distinctive characteristics of apoptotic cells were the presence of chromatin condensation/fragmentation and the acquisition of sub-G_1 DNA content. To examine chromatin structure, cells were fixed with ethanol, stained with DAPI, and examined by fluorescence microscopy. To measure DNA content, cells were permeabilized with Nonidet-P40, stained with PI, and the fluorescence analyzed by flow cytometry. As a routine, the capacity of the cells to exclude free penetration of trypan blue or PI was also examined, as an indication of plasma membrane integrity. These procedures were described in detail in preceding publications [28].

2.5. Determination of active Bax

Cells were fixed with 0.35% (v/v) formaldehyde and permeabilized with 0.1% (w/v) saponin in PBS for 5 min on ice. After incubation for 30 min at 4 °C with anti-Bax antibody clone 6A7, and for 30 min at 4 °C with FITC-conjugated anti-IgG antibody, the fluorescence was estimated by flow cytometry. The 6A7 clone recognizes a NH_2-terminal region of Bax which is occluded under normal conditions, but which is exposed as a consequence of changes in conformation associated to Bax translocation to mitochondria in stressed cells [29].

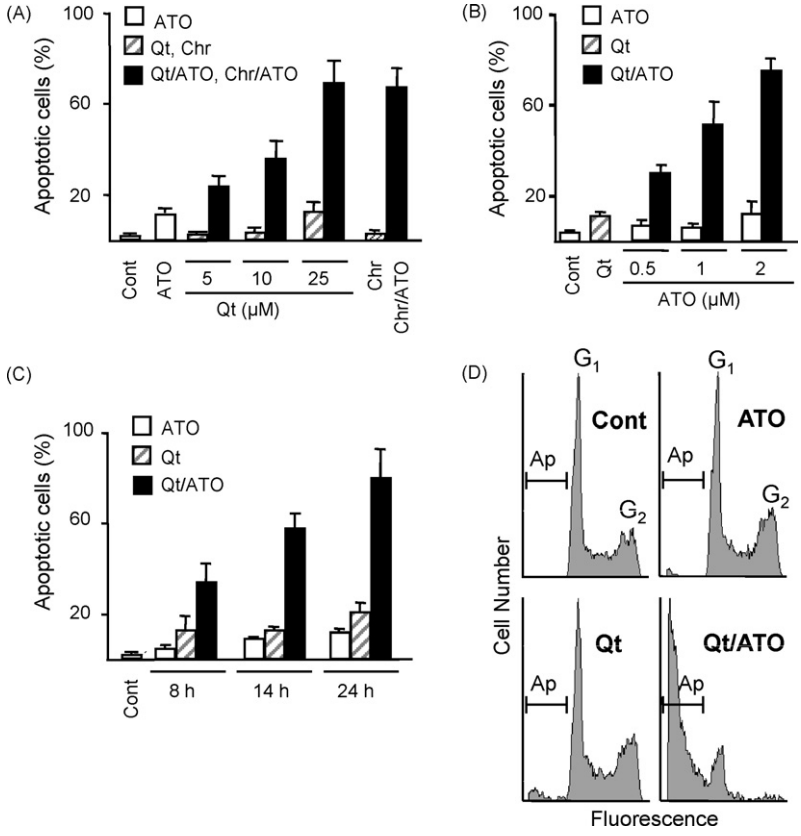

Fig. 1 – Induction of apoptosis by ATO, quercetin and chrysin in U937 cells. Panels (A)–(C) show the frequency of apoptotic cells, as determined by chromatin fragmentation, in (A) Untreated U937 cell cultures (Cont), cultures treated for 24 h with 2 μM ATO, cultures treated for 24 h with the indicated concentrations of quercetin (Qt), alone and in combination with ATO (Qt/ATO), and cultures treated for 24 h with 10 μM chrysin (Chr), alone and with ATO. (B) Cultures treated for 24 h with 25 μM quercetin, and cultures treated with the indicated concentrations of ATO, alone and with quercetin. (C) Cultures treated for the indicated time-periods with 2 μM ATO alone, 25 μM quercetin alone, and the combination of both drugs. The results show the mean ± S.D. of at least three determinations. (D) Cell cycle distribution and frequency of apoptotic cells (Ap), as determined by PI staining and flow cytometry analysis upon 24 h treatment with 2 μM ATO and 25 μM quercetin, alone and in combination. The profiles are indicative of one of two determinations with similar results.

2.6. Measurement of mitochondrial transmembrane potential

The mitochondrial transmembrane potential ($\Delta\Psi m$) was determined by flow cytometry after cell loading with rhodamine 123, as previously described [28].

2.7. Measurement of intracellular GSH content and GSH synthesis activity

The intracellular GSH content was determined by fluorometry after cell loading with monochlorobimane, following the previously described procedure [28].

The method used to measure the rate of GSH synthesis activity is an adaptation for human leukemia cells [26] of the procedure previously described by Fernández-Checa and Kaplowitz for human hepatocytes [30]. Namely, 5×10^6 cells were GSH-depleted by pre-incubation for 30 min with 0.8 mM maleic acid diethyl ester, after which they were extensively washed with Krebs-Henseleit buffer (NaCl 116 mM, KCl 5 mM, $CaCl_2$ 2.4 mM, $MgCl_2$ 1.2 mM, NH_2PO_4 1.2 mM, glucose 11 mM, pH 7.4), and re-suspended in 350 μl of the same buffer supplemented with 1 mM methionine, 1 mM serine, and 100 μM monochlorobimane. The increase in monochloribimane-derived fluorescence, which reflects the rate of GSH synthesis [30], was continuously recorded by fluorometry. This method indirectly provides an indication of BSO-inhibitable, γ-glutamylcysteine synthetase (γ-GCS) activity, the rate-limiting enzyme for GSH biosynthesis.

2.8. Immunoblot assays

To obtain total cellular protein extracts, cells were collected by centrifugation, washed with PBS, and lysed by 5-min heating at 100 °C followed by sonication in Laemmli's buffer containing a protease inhibitor cocktail, 10 mM sodium fluoride and 1 mM sodium orthovanadate. To obtain cytosolic extracts (aimed at determining cytochrome c and Omi/HtrA2 release from mitochondria) cells were collected for centrifugation, resuspended in 100 μl of ice-cold PBS containing 80 mM KCl, 250 mM sucrose, and 200 μg/ml digitonin, and kept on ice for 5 min. After centrifugation (10,000 × *g* for 15 min at 4 °C) the pellet was discarded. Fractions of the total or cytosolic extracts, containing equal protein amounts, were analyzed by SDS-polyacrylamide gel electrophoresis, blotted onto membranes, and immunodetected, as previously described [26].

3. Results

3.1. Apoptosis induction

Fig. 1A–C shows the capacity of quercetin and ATO, used alone and in combination, to cause apoptosis in U937 promonocytic cells, as determined by chromatin fragmentation. ATO was used at 0.5–2 μM, considered as clinically achievable concentrations [4]. Quercetin was applied at 5–25 μM, since these concentrations were earlier used with U937 [9] and other myeloid cell models [12]. At 24 h of treatment, quercetin started to cause detectable apoptosis at 25 μM, and ATO at 2 μM (approximately 15% apoptotic cells: see Fig. 1A and B). When used together, quercetin and ATO cooperated in more than additive manner to induce apoptosis at all assayed concentrations, with maximum efficacy at 2 μM ATO plus 25 μM quercetin (Fig. 1A and B). Using this combination, apoptosis was already detected at 8 h of treatment, and increased thereafter (Fig. 1C). Apoptosis still increased at treatments longer than 24 h, but prolonged treatments were usually omitted to avoid the appearance of secondary (apoptosis-derived) necrosis, which could mask the results. The cooperation between ATO and quercetin to induce apoptosis was corroborated by measuring the frequency of cells with sub-G_1 DNA content in flow cytometry assays (Fig. 1D). Moreover, the pan-caspase inhibitor z-VAD-fmk abrogated the toxicity of ATO plus quercetin (see Fig. 4F),

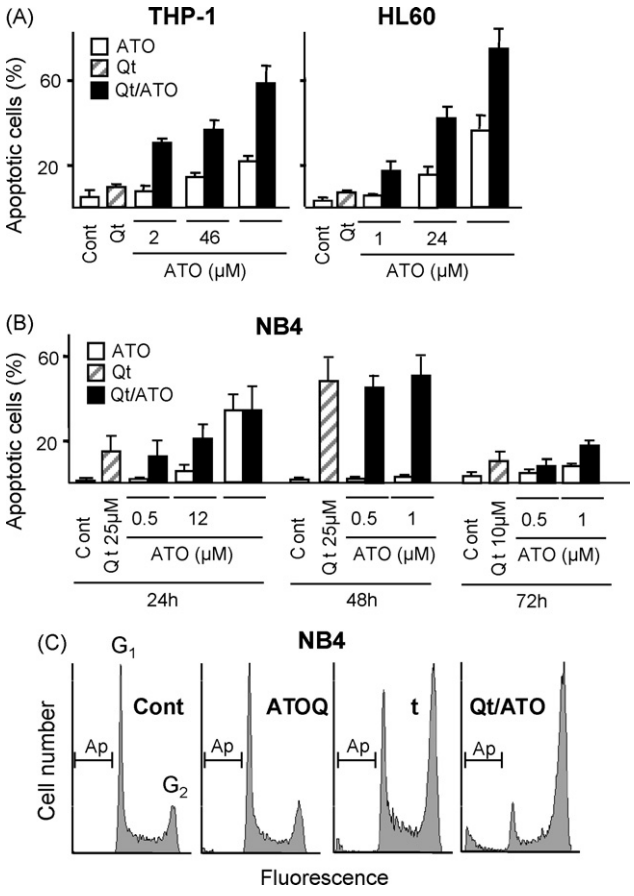

Fig. 2 – Apoptosis induction by ATO and quercetin in different leukemia cell lines. Panels (A) and (B) show the frequency of apoptotic cells, as determined by chromatin fragmentation, in (A) THP-1, HL60 cell cultures treated for 24 h with 25 μM quercetin, and with the indicated concentrations of ATO, alone and with quercetin; and (B) NB4 cell cultures treated for the indicated time-periods with the indicated concentrations of ATO and quercetin, alone and in combination. (C) Cell cycle distribution and frequency of apoptotic cells in NB4 cell cultures treated with 1 μM ATO and 25 μM quercetin, alone and in combination. All determinations were carried out at 24 h of treatment. All other conditions were as in Fig. 1.

indicating that the observed cell death is caspase-dependent apoptosis.

For comparison, determinations were carried out using the flavonoid chrysin instead of quercetin, and the leukemia cell lines THP-1, HL60 and NB4, instead of U937. It was observed that chrysin cooperated with ATO in inducing apoptosis in U937 cells with similar efficacy to quercetin (Fig. 1A). Quercetin and ATO cooperated in more than additive manner to induce apoptosis in THP-1 and HL60 cells (Fig. 2A), but the cooperative action was low (less than additive) in the case of NB4 cells. This later observation was corroborated using different concentrations of quercetin and ATO, and different treatment periods (from 24 to 72 h) (Fig. 2B). Of note, in NB4 cells quercetin greatly provoked cell accumulation at the G_2/M phase of the growth cycle (Fig. 2C), an effect which was less prominent in the case of U937 cells (see Fig. 1D) and the other assayed cell lines (result not shown).

Quercetin was occasionally reported to potentiate cytoreduction by cisplatin and ara-C in leukemia and other tumour cell models [12–15]. For this reason, determinations were carried out using combinations of those agents in U937 cells. For homogeneity with ATO, quercetin (10 or 25 μM, depending on the length of treatment) was simultaneously applied with cisplatin (3–8 μM for 24 h) or ara-C (5 and 10 μM for 24 h, and 0.01–1 μM for 48 h). As indicated in Fig. 3, quercetin did not cooperate with ara-C and cisplatin in inducing apoptosis, or the effect was lower than additive. Moreover, quercetin attenuated the toxicity of the highest cisplatin concentration (8 μM).

3.2. *Mechanisms of apoptosis execution*

ATO and quercetin have been characterized as mitochondria-targeting drugs, capable of binding the adenosine nucleotide translocator (ATO) [2] and interfering with mitochondrial respiration (ATO and quercetin) [3,16]. For this reason we wanted to analyze the dissipation of mitochondrial transmembrane potential ($\Delta\Psi m$), as a manifestation of mitochondrial dysfunction, as well as the behaviour of factors which regulate the mitochondrial executioner pathway. This included the release of mitochondrial proteins (cytochrome *c* and Omi/HtrA2) to the cytosol, the expression level of XIAP, the most potent member of the Inhibitor of Apoptosis Protein (IAP) family, and the expression or activation of the Bcl-2 family member proteins Bcl-2 and Bcl-X_L (anti-apoptotic) and Bax and Bid (pro-apoptotic), which regulate mitochondrial protein release [31]. In addition, we examined the behaviour of HSP70, since this protein prevents apoptosis by interfering with the mitochondrial executioner pathway [32], and HSP70 expression may be induced by ATO [25] and repressed by quercetin [33]. The results in Fig. 4 indicate that: (i) treatment with ATO or quercetin alone had little effect on $\Delta\Psi m$, but the combination of both drugs clearly elicited $\Delta\Psi m$ dissipation, as indicated by the decrease in rhodamine 123-derived fluorescence in flow cytometry assays (Fig. 4A). (ii) ATO and quercetin alone had little effect on mitochondrial protein mobilization and XIAP expression, but the combination clearly elicited cytochrome *c* and Omi/HtrA2 release from mitochondria (Fig. 4B) as well as XIAP down-regulation (Fig. 4C), as determined by immunoblot using cytosolic and total cellular extracts, respectively. (iii) Treatment with ATO plus quercetin did not affect total Bcl-2 expression, but caused a decrease in Bcl-X_L expression. The treatment also caused a decrease in the amount of Bid proform (23 kDa), which represents an indirect evidence of protein truncation/activation, required for this protein to acquire the pro-apoptotic capacity (Fig. 4C). (iv)

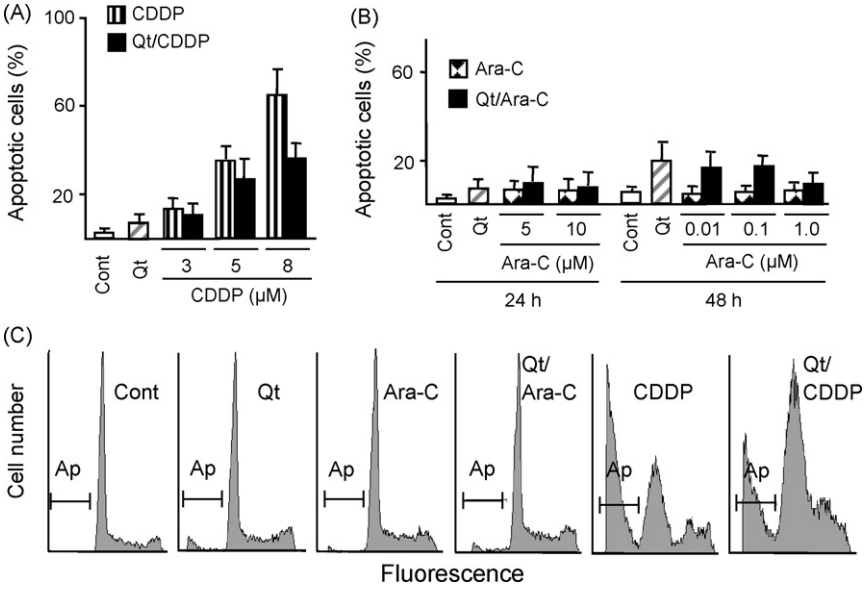

Fig. 3 – Apoptosis induction by quercetin, cisplatin and ara-C. Panels A and B show the frequency of apoptotic cells, as determined by chromatin fragmentation, in (A) U937 cell cultures treated for 24 h with 25 μM quercetin, alone and in combination with the indicated concentrations of cisplatin (CDDP) and (B) U937 cell cultures treated for 24 and 48 h with 25 and 10 μM quercetin, respectively, alone and in combination with the indicated concentrations of ara-C. (C) Cell cycle distribution and frequency of apoptotic cells in cultures treated for 48 h with 10 μM quercetin alone; for 48 h with 0.1 μM ara-C, alone and with 10 μM quercetin; and for 24 h with 8 μM cisplatin, alone and with 25 μM quercetin. All other conditions were as in Fig. 1.

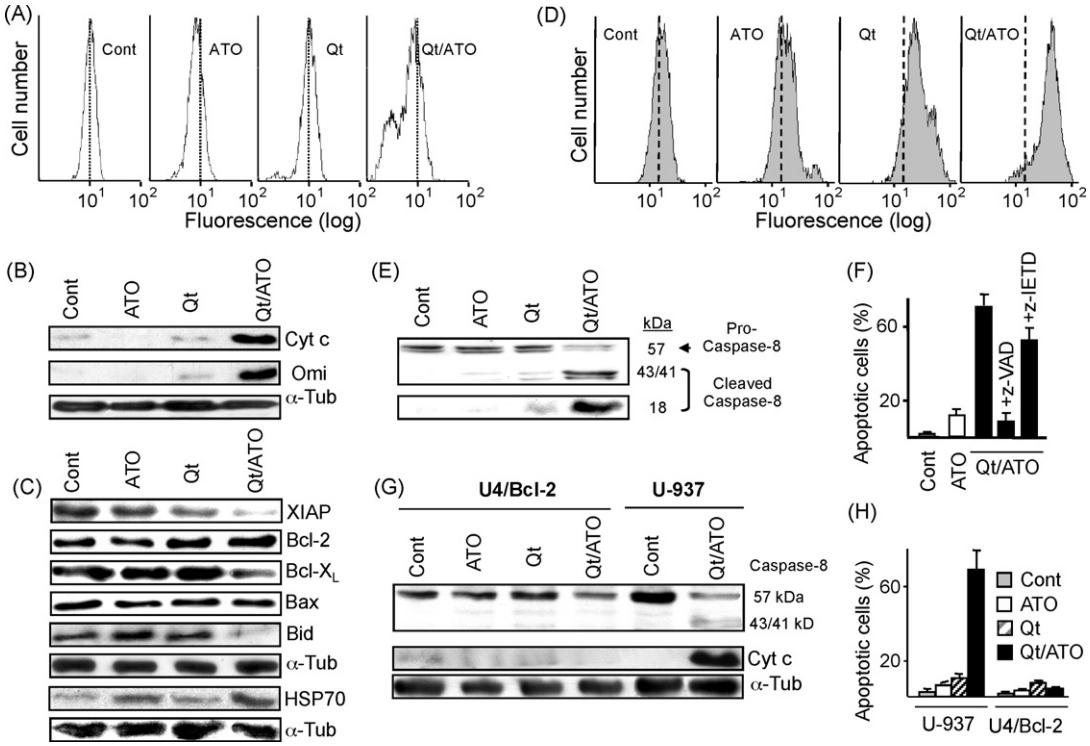

Fig. 4 – Disruption of mitochondrial transmembrane potential ($\Delta\Psi m$), and expression and processing of apoptosis-related proteins. U937 cell cultures were left untreated (Cont) or treated for with 2 μM ATO alone, 25 μM quercetin alone, and the combination of both drugs. (A) ($\Delta\Psi m$) dissipation, as determined by flow cytometry after cell loading with rhodamine 123. The vertical, dotted line represents the mean fluorescence value in controls, to better discern the displacement caused by the treatments. (B) Relative level of cytochrome c and Omi/HtrA2 in the cytosol, as determined by immunoblot using cytosolic extracts. The level of α-tubulin (α-tub) was also measured as a control. (C) Expression XIAP, Bcl-2, Bcl-X_L Bax, Bid (21-kDa proform), and HSP70, as determined by immunoblot using total cellular extracts. (D) Relative level of activated Bax, as determined by flow cytometry using the 6A7 antibody. (E) Cleavage-activation of pro-caspase-8, as evidenced by the disappearance of the 57 kDa pro-form and appearance of the 43/41 and 18 kDa cleavage fragments. (F) Effect of z-VAD-Fmk and z-IETD-fmk on ATO plus quercetin-produced apoptosis. The caspase inhibitors (50 μM) were applied 30 min before the other drugs. In these experiments, apoptosis was measured at 24 h of treatment, while all other determinations were carried out at hour 14. Pro-caspase-8 cleavage and levels of cytochrome c in the cytosol (G), and frequency of apoptotic cells (H), in Bcl-2-transfected (U4) cell cultures, as compared with non-transfected cells. In these experiments, all determinations were carried out at 24 h of treatment. The immunoblot and flow cytometry results are representative of one of at least two determinations. All other conditions were as in Fig. 1.

While ATO and quercetin failed to affect total Bax expression (Fig. 4C), they elicited Bax activation, and the effect was still higher when both agents were used in combination, as demonstrated by flow cytometry using the 6A7 antibody (Fig. 4D). (v) Finally, ATO increased HSP70 expression, but the increase was not prevented by co-treatment with quercetin (Fig. 4C).

The observation that Bid was cleaved in response to ATO plus quercetin led us to investigate the possible activation of caspase-8, since Bid is a substrate of this caspase. It was observed that ATO plus quercetin in fact caused caspase-8 cleavage/activation, as indicated by the decrease in 57 kDa pro-caspase content and the appearance of the 43 and 18 kDa cleavage-derived products (Fig. 4E). The caspase-8-specific inhibitor z-IETD-fmk attenuated apoptosis, although to much lower extent that the pan-caspase inhibitor z-VAD-fmk (Fig. 4F).

Enforced Bcl-2 expression is frequently used as a tool to prevent the activation of the mitochondrial apoptotic cascade, and to analyze the relationship between the mitochondrial and extrinsic (caspase-8-related) pathways [34, and references therein]. For this reason, experiments were carried out using a Bcl-2-transfected U937 cell clone (U4). Preliminary determinations indicated that U4 cells possess an approximately six- to eight-fold increase in Bcl-2 in comparison with non-transfected cells (result not shown). As expected, Bcl-2 over-expression blocked cytochrome c release (Fig. 4G), and also prevented apoptosis execution (Fig. 4H). Moreover, it could be observed that quercetin plus ATO failed to cause caspase-8 cleavage in U4 cells (Fig. 4G). This observation strongly suggests that caspase-8 activation in quercetin plus ATO-treated U937 cells is a secondary event derived from mitochondrial activation.

3.3. *Protein kinase activation*

Earlier reports demonstrated that the MAP kinases JNK, p38 and ERKs are important regulators of ATO toxicity, by playing either pro-apoptotic or anti-apoptotic roles [26,34–36]. In addition, other reports indicated that quercetin affects MAPK activation in association to apoptosis in tumour cells [18,37]. For these reasons, immunoblot assays were carried out to measure JNK, p38 and ERK phosphorylation, as an indication of kinase activation, in U937 cells treated with ATO and quercetin, alone and in combination. In addition, we examined the capacity 10 μM SP600125 (JNK inhibitor), 10 μM SB203580 (p38 inhibitor), and 2.5 μM U0126 (MEK/ERK inhibitor), to affect ATO plus quercetin-provoked apoptosis. The efficacy of these concentrations to prevent kinase activation in U937 cells was already proved in a preceding publication [35]. The results are indicated in Fig. 5A. JNK activation was only detected in ATO plus quercetin-treated cells at the latest assayed time (14 h). Treatment with ATO alone failed to activate ERK1/2 and p38, but these kinases were clearly activated by quercetin, alone and in combination with ATO, at all assayed times (Fig. 5A). Whatever the case, U0126, SB203580 and SP600125, failed to reduce the toxicity of ATO plus quercetin (Fig. 5B), indicating that kinase activation was irrelevant for apoptosis induction.

Earlier reports indicated that quercetin may inhibit Akt phosphorylation [8,14], and that down-regulation of the PI3K/Akt pathway increases ATO toxicity [25,38]. For these reasons, we asked whether the potentiation of apoptosis induction by ATO plus quercetin was associated to Akt inactivation. To examine the efficacy of quercetin as an Akt inhibitor in U937 cells, we performed a preliminary assay in which cells were

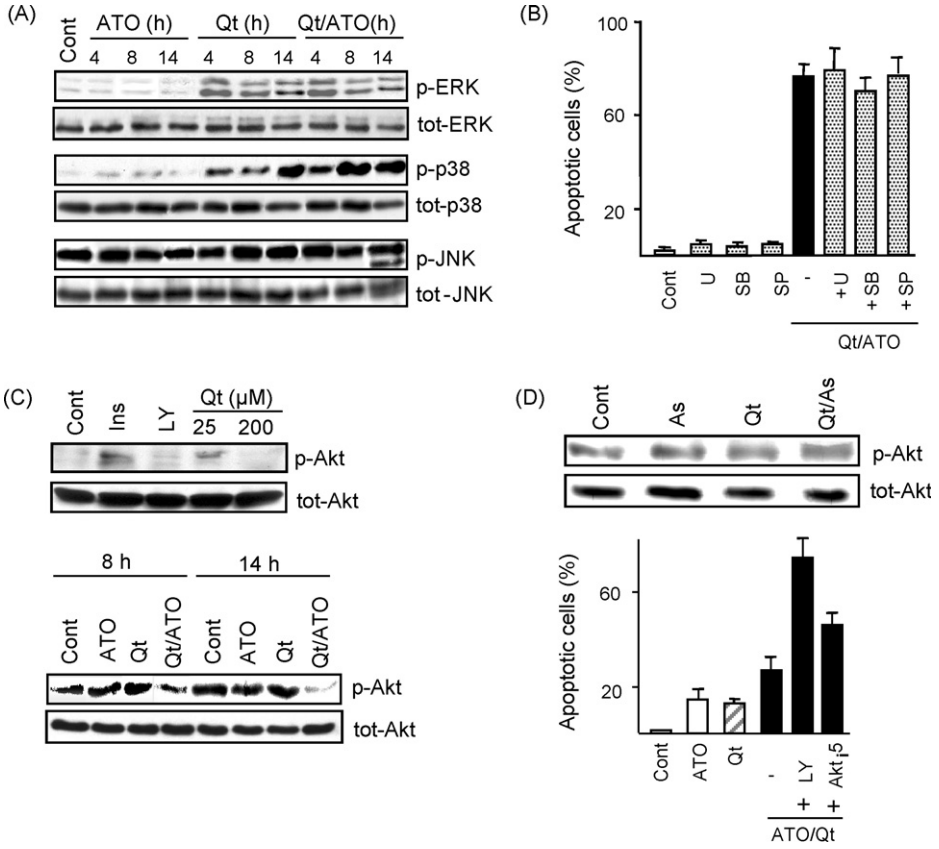

Fig. 5 – MAPK and Akt phosphorylation, and effect of MAPK inhibitors. (A) Relative levels of total (tot) and phosphorylated (p) ERK1/2, p38 and JNK, in U937 untreated cells (Cont) and cells treated for the indicated time-periods with ATO and quercetin, alone and in combination. (B) Frequency of apoptotic cells upon treatment for 24 h with 2.5 μM U0126, 10 μM SB203580, and 10 μM SP600125, alone and in combination with ATO plus quercetin. (C) The upper blot shows the level of total (tot) and phosphorylated Akt in serum-depleted, non-stimulated U937 cells (Cont) and cells stimulated for 15 min with 10 μg/ml insulin, in the absence (−) or the presence of 25 μM LY294002 (LY) or the indicated concentrations of quercetin. LY294002 and quercetin were added 1 h before insulin. The lower blot shows the levels of total and phosphorylated Akt in normally growing U937 cells at the indicated times of treatment with ATO and quercetin, alone and in combination. (D) The blot shows the level of total and phosphorylated Akt in NB4 cells treated for 24 h with ATO and quercetin, alone and in combination. The lower histogram shows the frequency of apoptotic cells in NB4 cell cultures treated for 24 h with 25 μM LY294002 or 25 μM Akt_iV, and with ATO and quercetin, alone or in combination with the PI3K/Akt inhibitors. ATO was used at 2 μM in U937 cells and 1 μM in NB4 cells, and quercetin was always used at 25 μM. The kinase inhibitors were always applied 30 min in advance to the other drugs. The immunoblots are representative of one of at least two determinations. All other conditions were as in Fig. 1.

firstly serum-starved to decrease the basal level of phosphorylated Akt, and then stimulated with insulin. The assay corroborated the capacity of the flavonoid to inhibit insulin-elicited Akt phosphorylation, albeit with lower efficacy than the canonical PI3K inhibitor LY294002, when used at equimolar concentration (25 μM) (Fig. 5C, upper blot). When assayed in normally growing non-stimulated cells, we failed to detect significant alterations in the constitutive levels of phosphorylated Akt upon treatment with quercetin alone, but the combination of both agents decreased Akt phosphorylation, which was detected from 8 h of treatment onwards (Fig. 5C, lower blot). On the other hand, the treatment did not apparently affect Akt phosphorylation in NB4 cells (Fig. 5D, upper blot), which correlates with the poor cooperation between ATO and quercetin in inducing apoptosis in this cell line (see Fig. 2B and C). Noteworthy, the administration of LY294002 or the Akt inhibitor Akt_iV increased the toxicity of ATO plus quercetin in NB4 cells (Fig. 5D, lower histogram), indicating that apoptosis induction in this cell line is sensitive to down-regulation of the PI3K/Akt pathway.

3.4. *Intracellular GSH content*

As indicated above, earlier studies demonstrated that ATO toxicity is strictly dependent on intracellular GSH content [21–24], that pharmacologic down-regulation of PI3K/Akt causes GSH depletion in myeloid leukemia cells [26], and that flavonoids may alter the intracellular GSH content [19,20]. For these reasons, fluorometric determinations using the GSH-sensitive probe monochloribimane were carried out to measure intracellular GSH in cells treated with ATO and quercetin, alone and in combination. The results are indicated in Fig. 6. Determinations at 24 h of treatment showed that 1–2 μM ATO did not cause significant alterations, that 25 μM quercetin alone decreased GSH, and that co-treatment with 1–2 μM ATO exacerbated the decrease produced by 10–25 μM quercetin (Fig. 6A). In addition, time-course studies indicated that ATO plus quercetin caused a progressive decrease in GSH content from 8 h onwards (Fig. 6B), thus paralleling the timing of apoptosis execution (see Fig. 1C). Importantly, the pan-caspase inhibitor z-VAD-fmk, which successfully prevented apoptosis execution (see Fig. 4F), failed to prevent GSH decrease (Fig. 6B). This excludes the possibility that GSH depletion in ATO plus quercetin-treated cells could be a trivial, secondary consequence of cell death. Chrysin also elicited GSH depletion, which was augmented in the presence of ATO (Fig. 6A). By contrast quercetin, alone and with ATO, failed to cause GSH depletion in NB4 cells (Fig. 6C), which correlates with the low cooperation of these agents to induce apoptosis in this cell line (see Fig. 2B and C). Of note, in spite of GSH depletion quercetin plus ATO did not significantly alter intracellular reactive oxygen species (ROS) generation in U937 cells, as measured at 1–14 h using the peroxide-sensitive fluorescent probe dichlorodihydrofluorescein diacetate (result not shown). Moreover, the toxicity of quercetin plus ATO was not affected by the addition of antioxidant or ROS-scavenging agents, namely catalase, tiron, trolox, and butylated hydroxyanisole (see Fig. 7C).

To shed light on the mechanisms accounting for GSH depletion as well as on the relationship between GSH

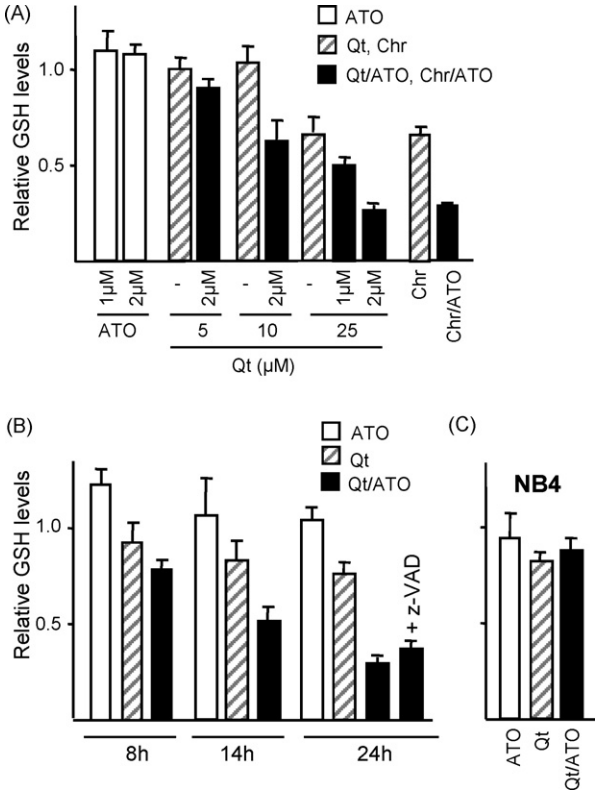

Fig. 6 – GSH depletion. The figure shows relative levels of intracellular GSH, as measured by monochlorobimane derivatization and fluorometric determination. All values are expressed in relation to untreated cultures (approximately 9 nmol/10^6 cells in U937 cells and 6 nmol/10^6 cells in NB4 cells), which received the arbitrary value of one. (A) GSH content in U937 cells treated for 24 h with the indicated concentrations of ATO, quercetin, and chrysin, alone and in combination. (B) GSH values in U937 cells treated for the indicated time-periods with 2 μM ATO and 25 quercetin, alone and in combination. In some determinations, 50 μM z-VAD-fmk was added 30 before ATO and quercetin. (C) GSH values in NB4 cells treated for 24 h with 1 μM ATO and quercetin, alone and in combination. All other conditions were as in Fig. 1.

depletion and apoptosis potentiation, we wanted to determine the effect of quercetin plus ATO on the rate of GSH synthesis under conditions with likely reflect γ-GCS activity (see Section 2). In addition, we measured the effects of NAC (10 mM) and BSO (1 mM) on GSH content and apoptosis. NAC was earlier employed as a cysteine donor for GSH biosynthesis in studies with ATO [21,24,26], and BSO is a potent inhibitor of γ-GCS activity, commonly used to experimentally deplete intracellular GSH [39]. At the used concentrations these drugs were not toxic for U937 cells. The results in Fig. 7A indicate that quercetin plus ATO elicited a decrease in the rate of GSH synthesis, of similar intensity to that provoked by BSO (included as control), suggesting that the treatment regulates GSH production at the level of enzyme activity. By contrast, NAC failed to restore the GSH content (Fig. 7B), and

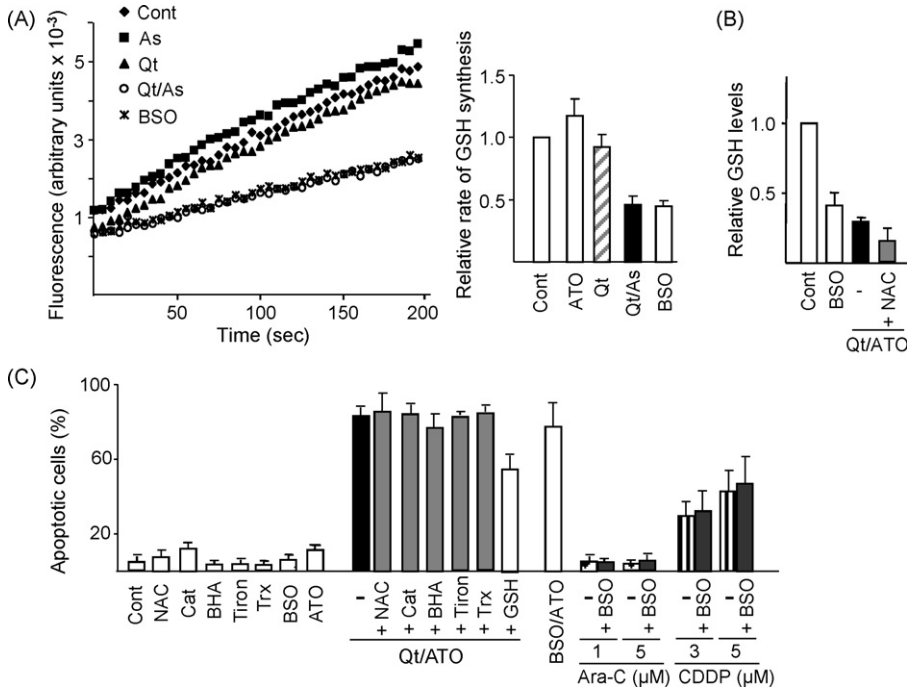

Fig. 7 – GSH synthesis, GSH depletion and apoptosis induction. (A) Relative rate of GSH synthesis in U937 cells, measured at 14 h of treatment with 1 mM BSO or with ATO and quercetin. Following GSH depletion with diethyl maleate, U937 cells were incubated with Krebs-Henseleit buffer supplemented with methionine, serine, and monochloribimane, and the fluorescence was continuously recorded, as an indication of the relative rate of GSH synthesis (see Section 2). The left panel shows the fluorescence values recorded in a representative determination. The right histogram shows relative RNA synthesis values (mean ± S.D.) calculated from five determinations. The results are expressed in relation to untreated cells, which received the arbitrary value of one. (B) Intracellular GSH content in U937 cells treated for 24 h with 1 mM BSO, or with ATO plus quercetin in the absence (−) or the presence of 10 mM N-actyl-L-cysteine (NAC). (C) Frequency of apoptosis in U937 cells treated for 24 h with ATO, BSO, NAC, and the antioxidants catalase (Cat, 500 U/ml), butylated hydroxyanisole (BHA, 100 μM), tiron (100 μM) and trolox (Trx, 100 μM); in cells treated with quercetin plus ATO in the absence (−) or the presence of NAC, 5 mM GSH-OEt (GSH), and the above-indicated antioxidants; and in cells treated with ATO plus BSO or with the indicated concentrations of ara-C and cisplatin, in the absence (−) or the presence of BSO. All agents were applied 2 h before ATO and quercetin. Except when otherwise indicated, ATO was used at 2 μM in U937 cells and 1 μM in NB4 cells, and quercetin at 25 μM. All other conditions were as in Fig. 1.

accordingly was unable to prevent apoptosis (Fig. 7C) in quercetin plus ATO-treated cells. As expected, BSO greatly decreased GSH content (Fig. 7B) and accordingly increased the ATO-provoked apoptosis (Fig. 7C). Noteworthy, co-treatment with BSO did not increase the apoptotic action of cisplatin and ara-C (Fig. 7C), which correlates with the low cooperative action between quercetin and these antitumour drugs (see Fig. 3).

As a complementary experiment, we analyzed the effect of GSH-OEt, earlier used to directly increase intracellular GSH in arsenate-treated cells [40]. It was observed that co-treatment with GSH-OEt attenuated the toxicity of quercetin plus ATO, which apparently supports the importance of GSH in regulating the apoptosis process. Nonetheless this result must be considered with caution, since possible interaction of ATO and GSH in the extra-cellular milieu may not be excluded [41]. Administration of GSH-OEt only as a pre-treatment caused a moderate (approximately 40%) increase in the constitutive intracellular GSH content, but unfortunately this increase disappeared soon after quercetin plus ATO administration (results not shown).

4. Discussion

The present work we demonstrates that the flavonoids quercetin and chryisin cooperate with the antileukemic agent ATO in inducing apoptosis in human leukemia cells. Nonetheless the efficacy of the response depended on the used cell line, with a maximum efficacy in U937 promonocytic cells, and less than additive effect in NB4 acute promyelocytic cells. In addition, the cooperative effect was null or negligible when U937 cells were co-treated with quercetin plus either ara-C or cisplatin, indicating a drug-selective response. Our observation is consistent with an earlier publication showing that quercetin did not affect ara-C cytotoxicity in HL60 and U937 cells [42], although other reports indicated cooperation between these drugs to inhibit cell proliferation [15]. Earlier studies indicated that the cooperation between quercetin and cisplatin in inducing apoptosis depended very much on the order of drug administration, the effect being low or null when the drugs were applied at the same time [12,13]. This is also consistent with our results, since in the present study all drugs

were simultaneously applied. An explanation for the different efficacy of quercetin to improve the apoptotic action of ATO and other antitumour drugs will be discussed later.

Although a detailed analysis of the apoptotic machinery was beyond the scope of this work, the present results indicate that quercetin and ATO cooperated in activating the mitochondria executioner pathway, which is consistent with the characterization of both agents as mitochondria-targeting drugs [2,3,16]. Two key events in this pathway are the release of cytochrome *c* from mitochondria to the cytosol, which is required for apoptosome assembly and subsequent activation of caspase-9/-3, and XIAP down-regulation, which relieves caspases from the inhibitory action exerted by that protein [31]. The potentiation of cytochrome *c* release by ATO plus quercetin is consistent with the decreased expression of Bcl-X_L (anti-apoptotic) and the increased activation of Bax (pro-apoptotic). XIAP down-regulation probably reflects an increase in protein degradation, since ATO plus quercetin elicited the release of Omi/AtrA2, and this protein was characterized as a serine protease which binds and degrades IAPs [43]. Moreover, IAPs are degraded by the ubiquitin-proteasome system [44], and earlier studies reported an increase in ubiquitin-conjugated proteins in arsenic-treated cells [45]. Of note, in addition to the mitochondrial pathway, co-treatment with quercetin plus ATO also succeeded in activating the caspase-8/Bid pathway. The possibility that caspase-8 activation by ATO is a death receptor-mediated event, or alternatively represents a secondary event derived from mitochondrial activation, was earlier analyzed in different leukemia cell models, with non-coincident results [36,46,47]. While this aspect was not directly approached in our study, the fact that Bcl-2 over-expression totally blocked caspase-8 cleavage strongly suggests that caspase-8 activation by ATO plus quercetin is downstream the mitochondrial pathway, at least in the U937 cell model. Moreover, the fact apoptosis is totally abrogated by Bcl-2 over-expression, but only partially reduced by the caspase-8 inhibitor, suggests that the caspase-8/Bid activation functions as an amplification loop for the final apoptotic result.

Finally, the present experiments indicated that co-treatment with quercetin plus ATO down-regulates Akt phosphorylation and decreases the intracellular GSH level in U937 cells, a result which agrees with our previous observations using ATO in combination with typical PI3K/Akt inhibitors [25]. Moreover, the observation that ATO plus quercetin reduced the γ-CCS-dependent GSH synthesis, while NAC was unable to restore the intracellular GSH level, suggests that quercetin plus ATO regulates GSH synthesis at the level of enzyme activity, as it was previously demonstrated in the case of PI3K inhibitors [26]. On the ground of the well-proved inverse relationship between ATO toxicity and GSH content [21–24], it may be reasonably proposed that GSH depletion explains at least in part the increased toxicity in quercetin plus ATO-treated cells. Since ATO detoxification is largely catalysed by glutathione S-transferases [48], intracellular GSH depletion may result in the increase in intracellular free ATO concentration, and hence in toxicity. Moreover, since arsenic readily reacts with vicinal cysteine residues of proteins [49], GSH depletion might cause a reduction in GSH-protein mixed disulfide formation, which in turn facilitates protein damage by ATO binding to unprotected cysteines. As a support for the importance of Akt inhibition and GSH depletion for apoptosis potentiation, the combination of quercetin plus ATO failed to down-regulate Akt phosphorylation and decrease GSH content in NB4 cells, and accordingly the cooperation to induce apoptosis was low in this cell line. Moreover, this hypothesis may provide a satisfactory explanation for the null or poor cooperative effect between quercetin and ara-C or cisplatin, since under the here used experimental conditions these agents behaved as GSH-insensitive drugs, as proved by the inability of BSO to potentiate ara-C- and cisplatin-provoked apoptosis. Of course, the present results do not exclude the possibility that factors other than the Akt/GSH pathway may participate in regulating the susceptibility to apoptosis. For instance, quercetin might directly inhibit the expression or activity of MRP1/2 drug efflux pumps, thus reducing drug detoxification [50]. In addition, the low cooperation between quercetin and ATO in NB4 cells correlated with a high capacity of the flavonoid to cause G_2/M blockade in this line, and G_2 arrest may temporarily restrain the cells from entering into apoptosis [51]. These and other possibilities are under investigation.

In summary, the present results indicate that quercetin selectively potentiates the apoptotic action of ATO in myeloid leukemia cells, an effect which may be explained at least in part by the capacity of the flavonoid by cause Akt down-regulation and GSH depletion. These observations may offer a rationale for the use of flavonoids to improve the clinical efficacy of ATO and perhaps other GSH-dependent antitumour drugs.

Acknowledgments

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