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COX-2 inhibitors block chemotherapeutic agent-induced apoptosis prior to commitment in hematopoietic cancer cells

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ABSTRACT

Enzymatic inhibitors of pro-inflammatory cyclooxygenase-2 (COX-2) possess multiple anti-cancer effects, including chemosensitization. These effects are not always linked to the inhibition of the COX-2 enzyme. Here we analyze the effects of three COX-2 enzyme inhibitors (nimesulide, NS-398 and celecoxib) on apoptosis in different hematopoietic cancer models. Surprisingly, COX-2 inhibitors strongly prevent apoptosis induced by a panel of chemotherapeutic agents. We selected U937 cells as a model of sensitive cells for further studies. Here, we provide evidence that the protective effect is COX-independent. No suppression of the low basal prostaglandin (PG)E_2 production may be observed upon treatment by COX-2 inhibitors. Besides, the non-active celecoxib analog 2,5-dimethyl-celecoxib is able to protect from apoptosis as well. We demonstrate early prevention of the stress-induced apoptotic signaling, prior to Bax/Bak activation. This preventive effect fits with an impairment of the ability of chemotherapeutic agents to trigger apoptogenic stress. Accordingly, etoposide-induced DNA damage is strongly attenuated in the presence of COX-2 inhibitors. In contrast, COX-2 inhibitors do not exert any anti-apoptotic activity when cells are challenged with physiological stimuli (anti-Fas, TNFα or Trail) or with hydrogen peroxide, which do not require internalization and/or are not targeted by chemoresistance proteins. Altogether, our findings show a differential off-target anti-apoptotic effect of COX-2 inhibitors on intrinsic *vs.* extrinsic apoptosis at the very early steps of intracellular signaling, prior to commitment. The results imply that an exacerbation of the chemoresistance phenomena may be implicated.

1. Introduction

Synthetic enzymatic inhibitors of the pro-inflammatory mediator cyclooxygenase-2 (COX-2) are pharmacological agents with important anti-cancer activities [1]. After the identification of the second inducible form of COX enzymes in the 1990s, numerous studies demonstrated that COX-2 is stably expressed in various cancers [2]. More detailed studies have described an aberrant constitutive COX-2 expression since the very early steps of carcinogenesis [3]. Accordingly, many *in vitro* and *in vivo* studies strongly suggested multiple pro-carcinogenic roles for COX-2 overexpression, ranging from the promotion of mutant cell proliferation to a causative role in determining chemotherapy failure favoring metastasis formation [1]. A consistent number of studies are based on the use of non-steroidal anti-inflammatory drugs (NSAIDs), which still represent the only available pharmacological approach to counteract COX-2 functions *via* inhibition of its enzymatic activity [1,4].

In some instances, COX-2 inhibitors affect cancer cell viability *per se* [5–7]; in other instances, these compounds sensitize cancer cells to other cytocidal treatments [8–10]. Sensitization to apoptosis has been demonstrated in the case of chemotherapeutic agents that activate the intrinsic (or mitochondrial) apoptotic pathway [10,11] as well as with agents that trigger the extrinsic (death receptor-mediated) apoptotic pathway (*i.e.*, TRAIL or TNFα) [8,12,13]. The published mechanisms appear quite heterogeneous. The disturbance of the pro-survival AKT-dependent pathway

Abbreviations: Bak, Bcl-2 homologous antagonist killer; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2; Bcl-Xl, B-cell lymphoma-extra large; CPT, camptothecin; CISP, cisplatin; COX-2, cyclooxygenase-2; CTR-1, copper transporter 1; CYT, cytarabine; DMC, 2,5-dimethyl-celecoxib; DOXO, doxorubicin; Fas, apoptosis stimulating fragment; γ-H2A.x, phosphorylated histone H2A.x; H_2O_2, hydrogen peroxide; IAP, inhibitor of apoptosis; IRINO, irinotecan; MDR-1, multidrug resistance protein 1; MRP-1, multidrug resistance-associated protein 1; MRP-6, multidrug resistance-associated protein 6; MTX, methotrexate; PMC, puromycin; NSAIDs, non-steroidal anti-inflammatory drugs; Rh 123, rhodamine 1,2,3; TNFα, tumour necrosis factor alpha; TRAIL, TNF-related apoptosis inducing ligand; VP16, etoposide; XIAP, X-linked inhibitor of apoptosis protein.

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[5,12], the counteraction of multi-drug resistance phenomena [14,15], an altered balance of the level of expression of anti-apoptotic *vs.* pro-apoptotic Bcl-2 family members [5,16] and the up-regulation [17] and promotion of clustering of death receptors [13] have been evoked to play a causative role. However, not all anti-cancer effects of synthetic COX-2 inhibitors may actually be ascribed to the inhibition of the COX-2 enzyme. Studies identifying the concentration of COX-2 inhibitors able to impact production of prostaglandins or studies based on the silencing of COX-2 gene expression by RNA interference-based approaches have not always confirmed the anti-cancer effects of COX-2 inhibitors, indicating the existence of COX-2-independent effects [1]. Some of these studies mention that the down-regulation of COX-2 expression is a factor that partially contributes but is not sufficient to completely describe the anti-cancer effects of COX-2 inhibitors [18,19]. The scenario is further complicated by the fact that the biological properties of COX-2 inhibitors sometimes appear to be confirmed by COX-2 gene down-regulation and sometimes not, even when the studies deal with the same COX-2 inhibitor [19,20]. The heterogeneity of the different cancer cell models used is one of the factors most often evoked to explain these contradictory results. Recently, a few studies have reported potential anti-apoptotic effects, which have so far been confined to specific conditions or compounds [20,21]. Nevertheless, a large body of evidence regarding the anti-cancer potential of COX-2 inhibitors has put these compounds at the centre of many investigations as a strategy to improve or develop further successful anti-cancer therapeutic approaches.

The data concerning the effects of COX-2/and COX-2 inhibitors on cancer cells has so far derived mainly from adherent cell models. More recently, evidence for a stable COX-2 expression was also found in leukemic/lymphoblastic cancers, where a similar pro-carcinogenic role of COX-2 has been hypothesized [22,23].

In this study, we investigated the effects of three COX-2 inhibitors (nimesulide, NS-398 and celecoxib) on apoptosis induced by a panel of cytocidal treatments. Here we show that all three inhibitors specifically counteract cell death induced by chemotherapeutic agents that trigger stress-mediated apoptosis but not by physiological stimuli, which act *via* death receptor activation. The differential effect on intrinsic *vs.* extrinsic apoptosis is a consequence of the ability of COX-2 inhibitors to prevent stress-induced apoptosis at the very early steps of the intracellular signaling, prior to commitment. This effect appears to be COX-2 independent.

2. Materials and methods

2.1. Materials

Nimesulide and NS-398 were purchased from Cayman Chemicals (Ann Arbor, MI, USA). Celecoxib was from Merck (Leuven, Belgium). Anti-Fas (clone CH11) was from Millipore, Upstate (Lake Placid, NY, USA). TNFα was purchased from Reliatech (Wolfenbüttel, Germany), Superkiller Trail was from Alexis Axxora (Zandhoven, Belgium). Etoposide, puromycin, hydrogen peroxide, doxorubicin, camptothecin, phorbol 12-myristate 13-acetate (PMA) were from SIGMA (Bornem, Belgium). Cisplatin and methotrexate were purchased from Teva Pharma Belgium (Wilrijk, Belgium), irinotecan and cytarabine were from Pfizer Pharmaceuticals (Bruxelles, Belgium).

2.2. Cell culture and treatments

U937 (human histiocytic lymphoma), Jurkat (acute lymphoid leukemic T cells), K562 (chronic myeloid leukemia), Raji (Burkitt's lymphoma), Hel (human megakaryocytic acute myeloid leukemia), cells (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, DSMZ, Braunschweig, Germany) were cultured in RPMI 1640 medium (Bio-Whittaker, Verviers, Belgium) supplemented with 10% (v/v) fetal calf serum (FCS; Lonza, Verviers, Belgium), 1% (v/v) antibiotic–antimycotic solution (Lonza, Verviers, Belgium) and 2 mM L-glutamine (Lonza). KBM5 (chronic myeloid leukemia) were kindly donated by Dr. Bharat B. Aggarwal and cultured in IMDM medium (Bio-Whittaker, Verviers, Belgium) containing 15% (v/v) fetal calf serum (FCS; Lonza). All the cell lines were kept at 37 °C in a 5% CO_2 humidified atmosphere. Cells were pre-treated for 24 h with nimesulide, NS-398 (0–100 μM) or celecoxib (0–40 μM) before other treatments.

2.3. Induction of apoptosis

Apoptosis was induced with: (a) stressing compounds: the topoisomerase II inhibitor etoposide (VP16; 100 μM, 4 h); the topoisomerase I inhibitors camptothecin (CPT; 10 μM, 8 h) and irinotecan (IRINO; 10 μM, 8 h), the alkylating agent cisplatin (CISP; 100 μM, 24 h); the DNA intercalating agent doxorubicin (DOXO; 4 μM, 8 h), the metabolite analogues methotrexate (MTX; 20 μM, 8 h) and cytarabine (CYT; 10 μM, 24 h); the protein synthesis inhibitor puromycin (PMC; 10 μg/ml, 5 h); and the oxidative stress inducer hydrogen peroxide (H_2O_2; 500 μM); (b) physiological stimuli: anti-Fas (clone CH11; 50 nM, 16 h); TNFα (1 ng/ml, 16 h); and Trail (0.5 μg/ml, 8 h). Freshly prepared H_2O_2 was added to the medium and incubated for 1 h at 37 °C; the cells were then washed and resuspended in fresh medium (recovery phase). All the other treatments were kept throughout the experiment.

2.4. Analysis of apoptosis

Apoptosis was measured by analysis of nuclear fragmentation (1 μg/ml Hoechst 33342) [24] and confirmed by the estimation of mitochondrial membrane potential loss by incubating 1×10^6 cells at 37 °C for 20 min with 50 nM MitoTracker® Red (MTR, Molecular Probes/Invitrogen, Merelbeke, Belgium) [24], followed by flow cytometric analysis (FACScalibur, BD Biosciences, San José, CA, USA).

2.5. RNA extraction and real-time PCR analysis

Total RNA from U937 and K562 cells was extracted as previously published [25]. One microgram of RNA was used for reverse transcription with oligo(dT) primers. cDNA products were used for PCR amplification with the Platinum® High Fidelity *Taq* DNA Polymerase (Invitrogen, Merelbeke, Belgium) and gene-specific primers for COX-2 (forward: 5′-GCCCAGCACTTCACGCAT-GAG-3′; reverse: 5′-AGACCAGGCACCAGACCAAAGACC-3′), MDR-1 (forward: 5′-CAGAGGGGATGGTCAGTGTT-3′; reverse: 5′-CCTGACTCACCACACCAATG-3′), MRP-1 (forward: 5′-CCTGTTCAACGTCATTGGTG-3′; reverse: 5′-AGCCACGTA-GAACCTCTGGA-3′), and β-actin was used as a control (forward: 5′-CTCTTCCAGCCTTCCTTCCT-3′) (reverse: 5′-AGCACTGTGTTGGCGTACAG-3′). All primers were from Eurogentec (Liege, Belgium). cDNA amplification was performed for 40 cycles with the following settings: 94 °C for 2 min, 60 °C for 1 min and 68 °C for 2 min. Results were expressed as the ratio of mRNA of target gene/mRNA β-actin.

2.6. Indirect immunofluorescence

U937 cells were fixed/permeabilized and immunostained as described [24]. The following parameters were analyzed: (a) mitochondrial cytochrome *c* release [26]; (b) Bax/Bak status activation (monoclonal mouse anti-Bax 6A7; Santa Cruz

Biotechnology, Boechout, Belgium; monoclonal mouse anti-Bak AB-1; Calbiochem, Leuven, Belgium); (c) Bax translocation to mitochondria by co-immunostaining U937 cells with the polyclonal rabbit anti-Bax (Δ-21; Santa Cruz Biotechnology) and anti-cytochrome c oxidase IV (COX IV, Santa Cruz Biotechnology) [27]; (d) DNA damage assessment, estimated by phosphorylation of histone H2A.x using the mouse monoclonal anti-γH2A.x (ser139; Millipore, Upstate) [28]; (e) multidrug resistance protein expression, MDR-1 (BD Biosciences Pharmingen, Erembodegem, Belgium) and MRP-1 (Santa Cruz Biotechnology). *In situ* analysis of immunostained cells include: (a) observation by fluorescence microscopy (Olympus, Hamburg, Germany). The images were analyzed and elaborated using the cell Cell^M software (Olympus Soft Images Solutions GMBH, Münster, Germany); (b) flow cytometric analysis. Events were recorded statistically (10,000 events/sample) using the CellQuest software (http://www.bdbiosciences.com/features/products/display_product.php?keyID=92). Data were further analyzed by using FlowJo software (http://www.flowjo.com/index.php).

2.7. Measurement of PGE_2 concentration

Prostaglandin E_2 (PGE_2) levels in cell culture supernatants were determined by using the prostaglandin E_2 EIA kit (Cayman Chemicals, Ann Arbor, MI, USA), which is based on competitive enzyme immunoassay using PGE_2 coupled with acetylcholinesterase as a tracer, according to the manufacturer's instructions. Briefly, the cells were seeded at a concentration of 0.5×10^6 cells/ml. After 24 h of treatment with COX-2 inhibitors, cells were centrifuged (350 g) and 50 μl of supernatant was collected. The concentrations of PGE_2 were determined according to a standard curve (in pg/ml) and were normalized by cell concentration. As a positive control, K562 cells treated for 24 h with 160 nM of phorbol 12-myristate 13-acetate (PMA, SIGMA) were used [29].

2.8. Drug efflux assay

U937 cells (10^6/ml) were incubated with 10 nM rhodamine 1, 2, 3 (Rh 123, SIGMA) for 30 min at 37 °C in normal culture conditions. Then, the fluorescent dye was washed out and the cells were seeded in fresh complete medium (recovery). COX-2 inhibitors were added again. Fluorescence was evaluated immediately ($T = 0$ h) and after 3 h ($T = 3$ h) of recovery time by flow cytometer analysis with FL2 (585/42 nm). The extent of drug efflux was calculated as a percentage of reduction of Rh 123 fluorescence (1-Rh 123 fluorescence ratio $T = 3$ h/$T = 0$ h) for each sample.

2.9. Western blot analysis

Protein separation by gel electrophoresis, protein transfer to nitrocellulose membranes and immunoblotting were performed as previously detailed [24]. Equal loading of samples was performed using β-actin as a control. A total of 5 μg of mouse macrophage lysate (BD Transduction Laboratories, Erembodegem, Belgium) co-stimulated with 10 ng/ml interferon γ(IFNγ) and 1 μg/ml lipopolysaccharide (LPS) was used as a positive control for COX-2 expression, according to the manufacturer's instructions. Primary antibodies: mouse monoclonal anti-β-actin, mouse monoclonal anti-caspase-3, goat polyclonal anti-COX-2, rabbit anti-CTR1 (Santa Cruz Biotechnology), anticaspase-8, anti-caspase-9 (Cell Signaling, Leiden, The Netherlands), anti-Bcl-xL (BD Transduction Laboratories), anti-Bcl-2 (Calbiochem, Leuven, Belgium). Incubation with the corresponding secondary antibodies (diluted in a PBS–Tween solution containing 5% of bovine serum albumin, BSA, or 5% of milk) was performed according to the manufacturer's instructions (HRP conjugated donkey anti-goat, goat anti-rabbit or goat anti-mouse from Santa Cruz Biotechnology). Specific immunoreactive proteins were visualized by autoradiography using the ECL Plus Western Blotting Detection System Kit (GE Healthcare, Roosendaal, The Netherlands).

2.10. Statistical analysis

Data are expressed as means ± SD, and the significance degree was analyzed by the Student's *t*-test. *p*-values below 0.05 were considered statistically significant.

3. Results

3.1. COX-2 inhibitors prevent stress-induced apoptosis without affecting apoptosis induced by physiological stimuli

U937 cells (Fig. 1A) were incubated for 24 h with different concentrations (10–100 μM) of one of the two COX-2 inhibitors nimesulide or NS-398. Then, cells were challenged with the chemotherapeutic agent etoposide (VP16; 100 μM). Both inhibitors did not impact cell viability *per se* but they prevented VP16-induced apoptosis in a dose-dependent manner, as determined by the analysis of nuclear morphology (Fig. 1B and C) and confirmed by the detection of caspase-3 cleavage (Fig. 1D and E).

To exclude that this effect was specific for VP16, we challenged U937 cells with different agents. Six chemotherapeutic agents, which trigger the intrinsic apoptotic pathway *via* different mechanisms (see Section 2), resulted strongly inhibited in their action by nimesulide comparable to VP16 (Fig. 2A); conversely, when cells were challenged with anti-Fas (50 nM), TNFα (1 ng/ml) or Trail (0.5 μg/ml), which initiate the extrinsic apoptotic pathway, COX-2 inhibitors did not play any modulating role (Fig. 2B). Similar results were observed with NS-398 (data not shown).

Since U937 cells stably express COX-2 (Fig. 1A), we investigated whether the anti-apoptotic effect depends on the inhibition of COX-2 enzyme activity or whether it was the consequence of an off-target effect. To address the question, first, we analyzed if the selective COX-2 inhibitor celecoxib, structurally unrelated to nimesulide and NS-398 [1] might prevent also apoptosis; besides, we tested the effect of its analog 2,5-dimethyl celecoxib (DMC) on apoptosis. This compound lacks the COX-2 inhibitory activity [30]. In U937 cells, incubated for 24 h with celecoxib (in the non cytotoxic range of 10–40 μM), then challenged with 100 μM VP16, the resulting apoptosis was prevented in a dose-dependent manner (Fig. 3A). DMC appeared toxic *per se* when used at concentrations >20 μM; when tested below this threshold, it similarly prevented apoptosis (Fig. 3B). Second, we assayed the amount of PGE_2 synthetized in U937 cells in the presence/absence of different concentrations of nimesulide, NS-398 or celecoxib. Fig. 3B shows that the low basal PGE_2 levels were not significantly affected by the incubation with the COX-2 inhibitors, even when they were used at the highest concentrations. Our results suggest that COX-2 even if expressed it is not enzymatically active in U937 cells.

Taken together, these results indicate differential abilities of COX-2 inhibitors in modulating intrinsic *vs.* extrinsic apoptotic pathways and strongly suggest that the protecting effect in stress-induced apoptosis is due to an off-target mechanism.

3.2. COX-2 inhibitors prevent stress-induced apoptosis by acting at the very early steps of the apoptotic signaling pathway

Next, we investigated at which step within the intrinsic apoptotic signaling cascade COX-2 inhibitors interfered in U937 cells. The induction of apoptosis by VP16 was chosen as a model. Moving backwards along the pathway [31], we found that the cleavage/activation of the effector caspase-9 was prevented (Fig. 4A); the same was observed for caspase-8, normally also

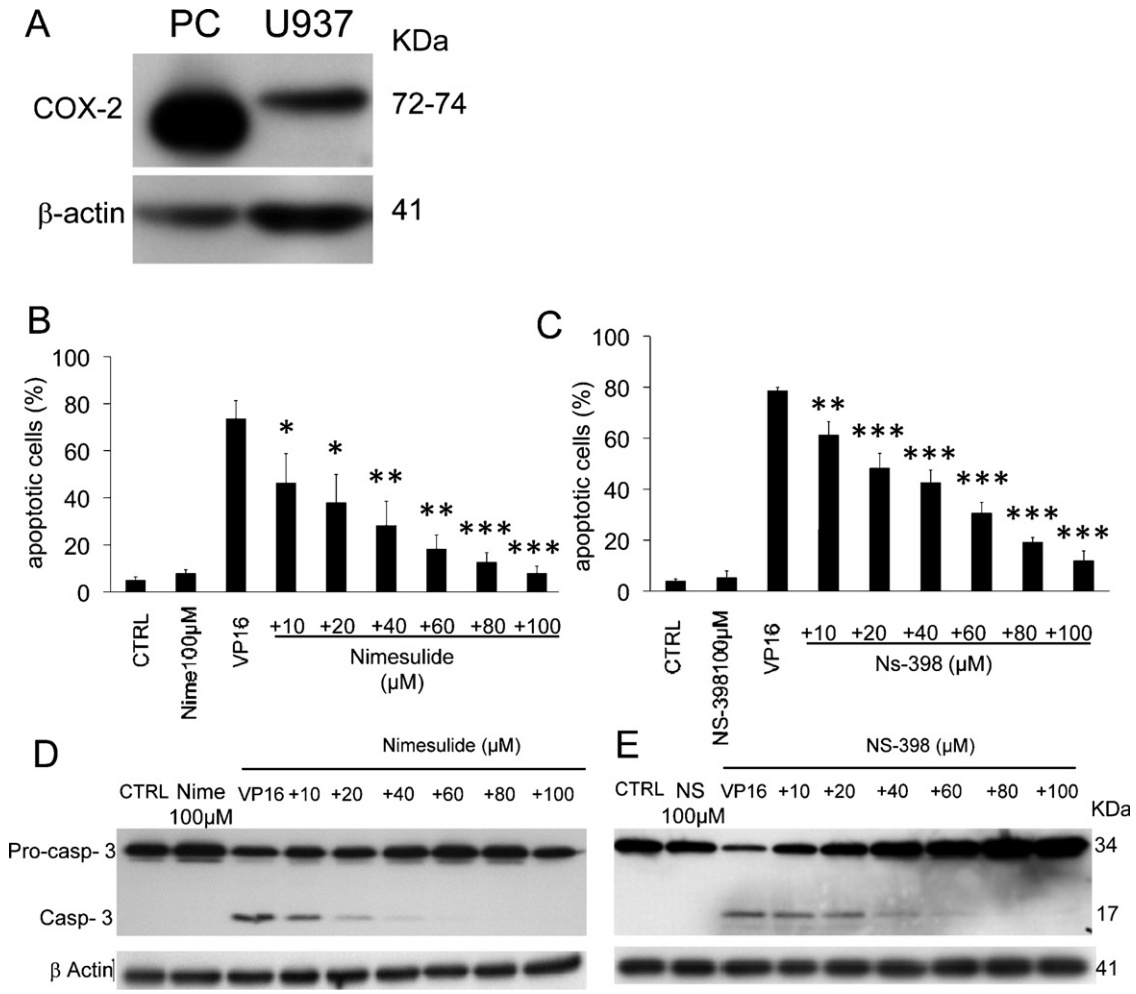


Fig. 1. COX-2 inhibitors prevent VP16-induced apoptosis in COX-2-positive U937 cells. (A) Assessment of COX-2 protein expression level in U937 cells (Western blot analysis). 20 μg of total protein extract was loaded along with 5 μg protein extracts of positive control (mouse macrophages stimulated with 10 ng/ml IFNγ/1 μg/ml LPS). One of three independent experiments with similar results is shown. Estimation of apoptosis by the analysis of nuclear morphology after Hoechst staining in U937 cells untreated or pre-treated for 24 h with 10–100 μM nimesulide (B) or NS-398 (C). Data represent the mean of five independent experiments ±SD. Significant difference compared to VP16-treated cells: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (D–E) Confirmation of the effects of COX-2 inhibitors on apoptosis by analysis of caspase-3 cleavage. One of three independent experiments was shown.

cleaved during VP16-triggered apoptosis (not shown) [32]. Therefore, we assessed the impact of COX-2 inhibitors on mitochondria by analyzing the mitochondrial membrane potential and the cytochrome *c* release. Both phenomena were inhibited in a dose-dependent manner (Fig. 4B and C). Similarly, the upstream activation of the two pro-apoptotic Bcl-2 family members Bax and Bak was impaired (Fig. 4D and supplemental Fig. 1).

Bax activation during apoptosis is a multi-step process, which includes translocation to mitochondria [33], an event that may be blocked even when Bax conformational change occurs. The pattern of intracellular distribution of Bax (independent of its activation status) appeared dotted in VP16-treated cells as expected [34], and overlapping with the mitochondrial protein COX IV (Fig. 5E), as the consequence of Bax re-localization to mitochondria [34]. The co-treatment with COX-2 inhibitors restored a diffuse pattern of Bax, comparable to control cells. The prevention of Bax/Bak functions is also not due to up-regulation of Bcl-2 anti-apoptotic members Bcl-2 or Bcl-xL (supplemental Fig. 2).

COX-2 inhibitors do not affect physiological stimuli (Fig. 2C). The mitochondrial pathway may be initiated downstream of caspase-8 activation [35] as part of an amplification signal. Thus, physiological stimuli like anti-Fas may lead to the activation of Bax and Bak, as a consequence of a cross talk occurring between the extrinsic and the intrinsic apoptotic pathway [36]. We investigated whether COX-2 inhibitors may affect the levels of Bax/Bak activation upon anti-Fas stimulation. Results show comparable levels of Bax and Bak activation in treated and untreated cells (supplemental Fig. 3). This finding also excludes direct modulations of Bax/Bak by COX-2 inhibitors and strongly suggests that COX-2 inhibitors act at very early steps of apoptotic signaling, likely at the commitment step.

3.3. COX-2 inhibitors prevented DNA damage and affected drug accumulation

The fact that COX-2 inhibitors specifically inhibit stress-induced apoptosis at very early steps may be the consequence of their ability to affect drug internalization or metabolism. This hypothesis may also explain why the apoptotic physiological stimuli, by acting on extracellular targets, are not affected. Accordingly, COX-2 inhibitors might reduce intracellular damage induced by chemotherapeutic agents as a consequence of their reduced intracellular availability. VP16 is a DNA damaging agent whose impact on DNA may be indirectly evaluated by histone H2A.x phosphorylation (γ-H2A.x) [37]. Fig. 5A (left panels) shows a typical time-course of H2A.x phosphorylation upon VP16

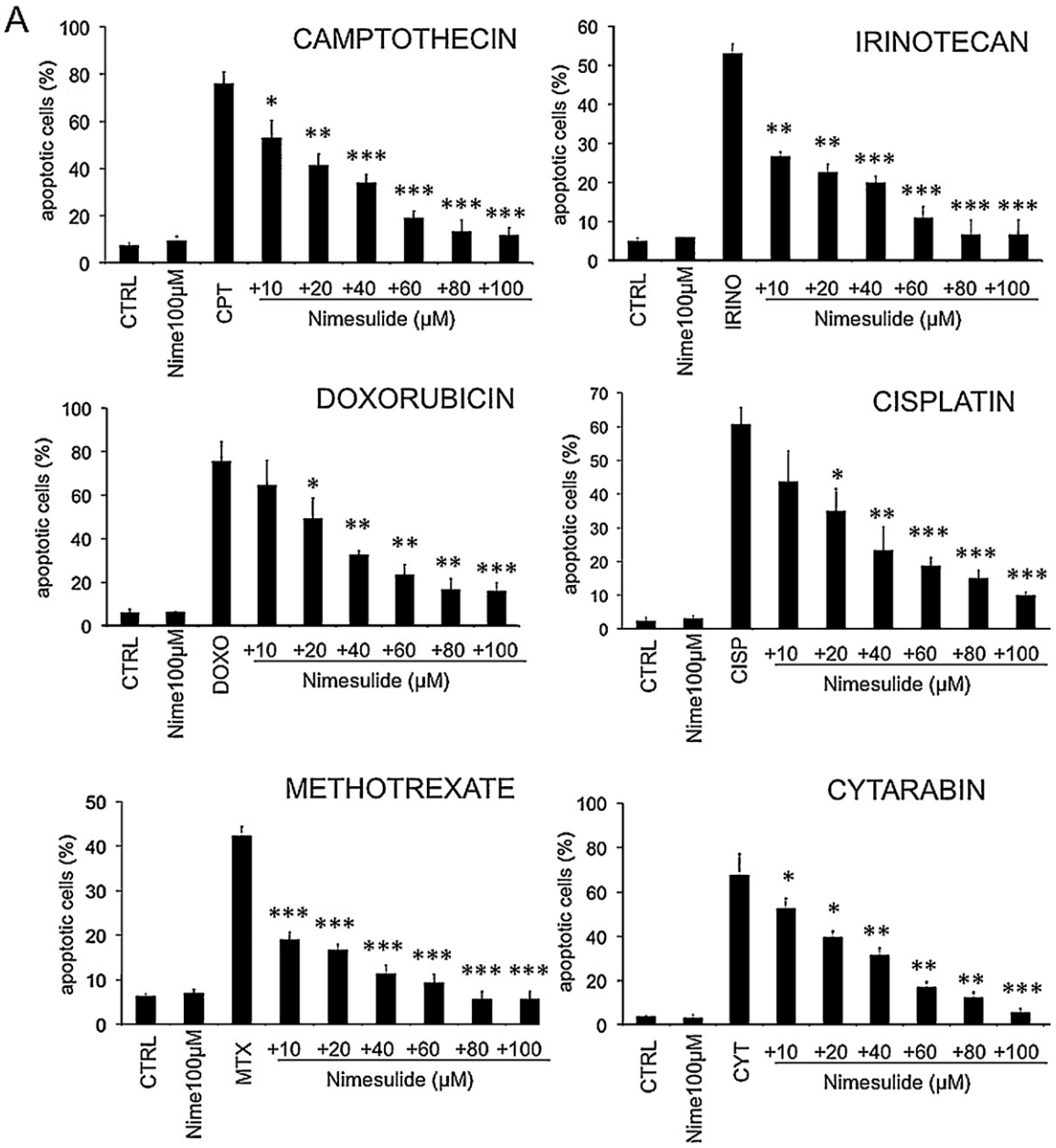


Fig. 2. Nimesulide protects U937 cells exclusively from stress-induced apoptosis. Effects of nimesulide on apoptosis induced by: (A) chemotherapeutic agents triggering the intrinsic apoptotic pathway: camptothecin (10 μM; CPT); irinotecan (10 μM; IRINO); doxorubicin (4 μM; DOXO); cisplatin (100 μM; CISP); methotrexate (20 μM; MTX); or cytarabine (10 μM; CYT) in combination with 0–100 μM nimesulide; (B) physiological stimuli: anti-FAS (50 ng/ml); TNFα (1 ng/ml) and TRAIL (0.5 μg/ml). The percentage of apoptotic cells was estimated by analysis of nuclear fragmentation. Data represent the mean of at least three independent experiments ±SD. Significant difference compared to VP16-treated cells: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Similar results achieved with NS-398 (not shown).

treatment. VP16-induced histone H2A.x phosphorylation was strongly prevented in the presence of nimesulide (Fig. 5A, right panels). The inhibition of γ-H2A.x accumulation continued even after longer incubation times with VP16, excluding the hypothesis that DNA damage was simply delayed. The impact of the other NSAIDs on VP16-induced DNA damage confirmed a similar pattern of modulation. Fig. 5B reports the quantification of cells positive to H2A.x phosphorylation in the control and in the pre-treated cells with each COX-2 inhibitor upon VP16 challenge.

Recently, the ability of celecoxib to modulate the drug importer CTR-1 was reported [21]. This inhibition counteracts the cytocidal activity of cisplatin in human esophageal squamous cancer cells [21]. Therefore, we assessed the ability of our panel of COX-2 inhibitors to modulate this carrier. Analysis by Western blot did not show any relevant impact on CTR-1 protein expression (Fig. 6A), thus excluding a relevant role in the phenomenon at least for this importer.

The anti-apoptotic effect of nimesulide is strongly limited when apoptosis is induced with the protein synthesis inhibitor puromycin in comparison to VP16 (supplemental Fig. 4). This result suggests that a neosynthesis, rather than a down-regulation, of protein factors may be implicated in effectively counteracting apoptosis.

Because one of the main reasons for chemotherapy failure is the exacerbation of events mediating drug efflux, we investigated if COX-2 inhibitors might promote drug extrusion. To address this question, we first performed a classical drug efflux assay based on the use of the fluorescent tool rhodamine 1,2,3 (Rh 123) [38] on U937 cells, either untreated or treated with 10, 40 or 100 μM of nimesulide or NS-398; alternatively, with 20 or 40 μM celecoxib. A consistent dose-dependent increase in drug efflux was observed

after nimesulide and NS-398 treatment, reaching maximum values

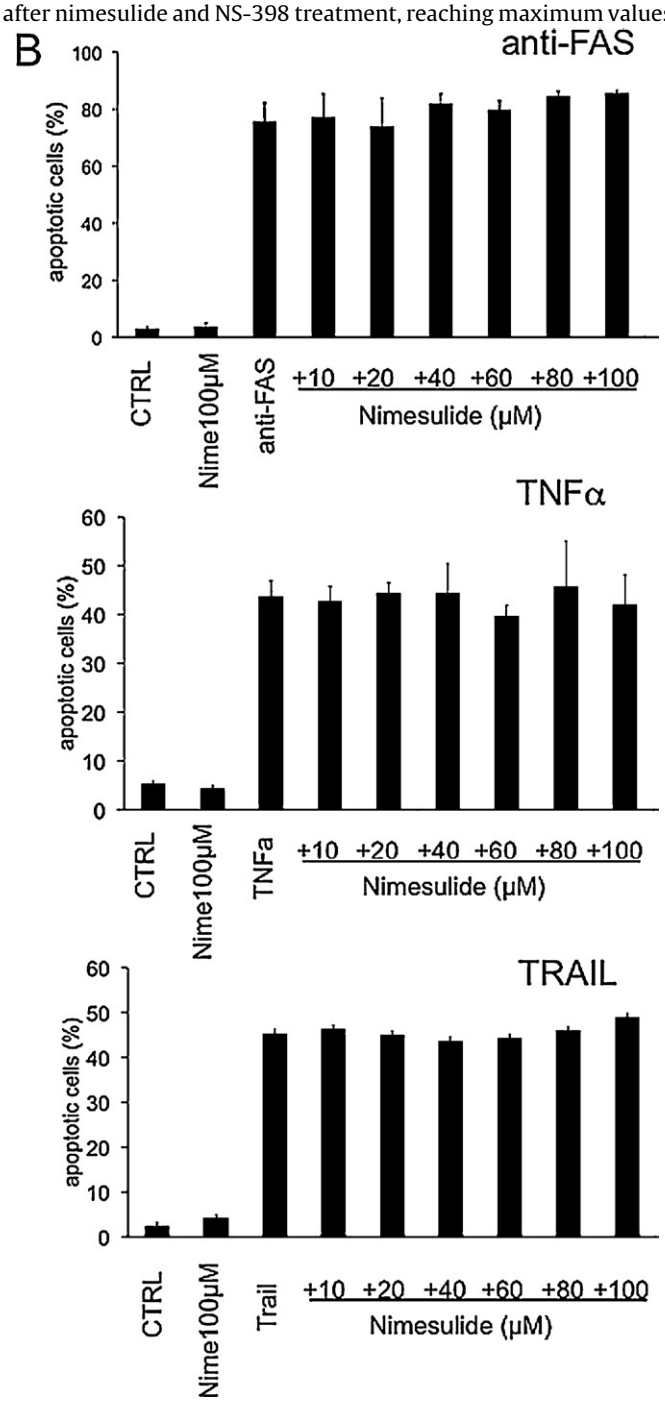


Fig. 2. (*Continued*).

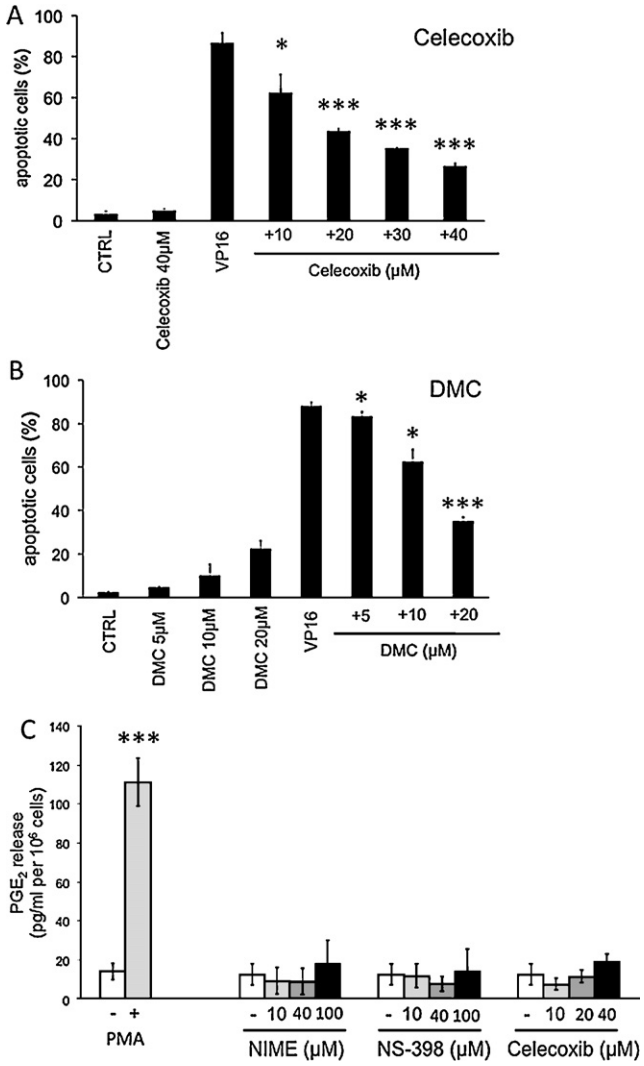
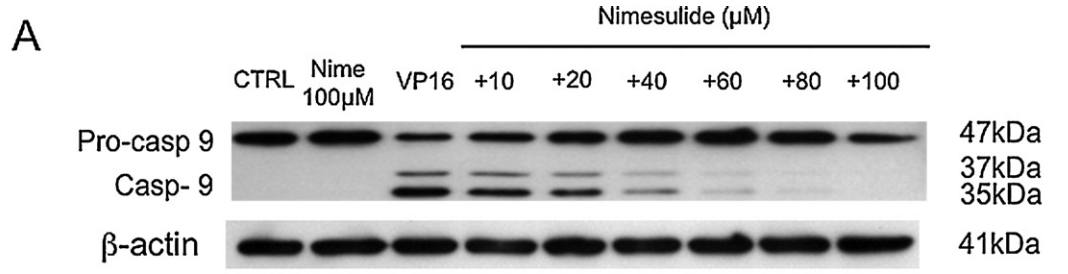


Fig. 3. The anti-apoptotic effect of COX-2 inhibitors is COX-independent. Estimation of percentage of apoptosis after Hoechst staining of U937 cells, either untreated or pre-treated 24 h with celecoxib (A) or DMC (B). Significant differences when compared to VP16-treated cells: $^{*}p < 0.05$, $^{***}p < 0.001$. (C) Quantification of PGE_2 levels from the supernatant of U937 cells incubated or not with the indicated concentrations of the nimesulide (NIME), NS-398 or celecoxib. The estimation of PGE_2 levels in K562 cells stimulated with PMA (see Section 2) was used as positive control (dashed column) in comparison to untreated K562 cells (first white column) and untreated U937 cells. PGE_2 is reported in pg/ml normalized for cell concentration (10^6 cells). Data are represented as the mean of four independent experiments ±SD.

of 45.80% ± 8.3 and 51.56% ± 6.60 reduction in fluorescence with 100 μM nimesulide or NS-398, and during the first 3 h of recovery, respectively (Fig. 6B). Celecoxib significantly increased drug efflux only at the concentration of 40 μM (14·6% ± 3.2 reduction), however at much lower values than those detected with nimesulide and NS-398. Next, we compared the expression levels of the most ubiquitous multidrug resistance proteins MDR-1 and MRP-1 [39,40] on the same cells. Supplemental Fig. 5B indicates a dose-dependent up-regulation at the mRNA levels for all the three different multi-drug carriers, with MDR-1 the most affected (MDR-1 = +3.09 ± 1.02, MRP-1 = +1.55 ± 0.25 with 100 μM nimesulide; MDR-1 = +3.83 ± 0.11, MRP-1 = +2.11 ± 0.11 with 100 μM NS-398). MDR-1 mRNA was also up-regulated by celecoxib in U937 cells (up to +34.74 ± 16.95 fold; supplemental Fig. 5C). However, the same analysis did not confirm any up-regulation of the corresponding protein (with MDR-1 paradoxically being even significantly decreased) (Fig. 6, panels C and D). These results indicate a differential ability of COX-2 inhibitors to impact drug efflux and exclude that these two main multidrug resistance proteins are implicated. Altogether, these findings exclude that drug efflux maybe the main mechanism responsible for the prevention of cell damage induced by chemotherapeutic agents.

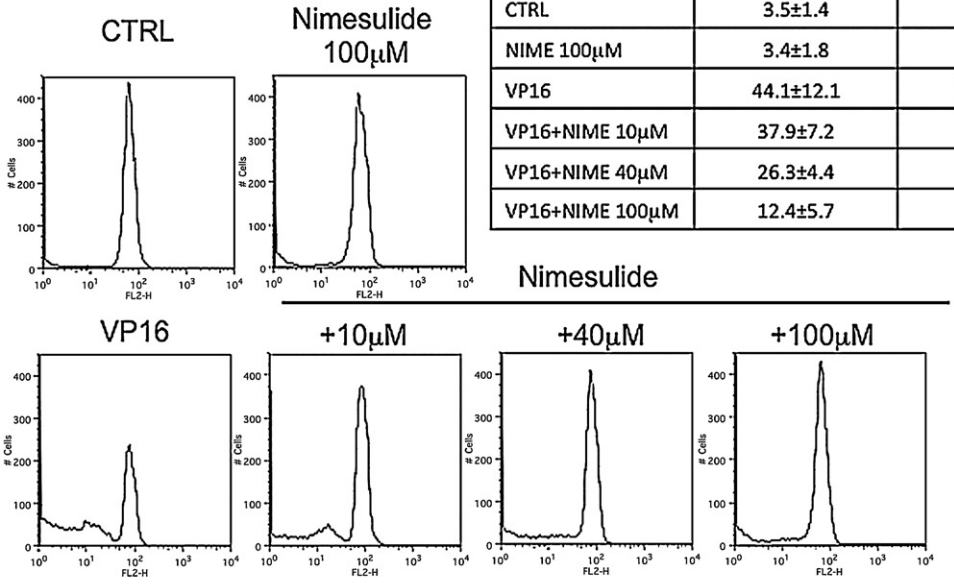
3.4. The ability of COX-2 inhibitors to counteract stress-induced apoptosis ubiquitously occurs in different hematopoietic cancer cell lines

So far, we have assessed a general ability of COX-2 inhibitors to counteract stress-induced apoptosis in U937 cells. We wanted to determine if this effect was ubiquitous. With this purpose, we



B

	% cells with low Δψ	% cells with high Δψ
CTRL	3.5±1.4	96.4±1.3
NIME 100μM	3.4±1.8	96.5±1.7
VP16	44.1±12.1	55.9±12.0
VP16+NIME 10μM	37.9±7.2	62.1±7.2
VP16+NIME 40μM	26.3±4.4	73.4±4.5
VP16+NIME 100μM	12.4±5.7	87.6±5.6



C

	% cells with released cytochrome c
CTRL	1.9±0.5
NIME 100μM	3.2±2.2
VP16	39.5±15.0
VP16+NIME 10μM	22.6±10.7
VP16+NIME 40μM	10.7±8.1
VP16+NIME 100μM	4.7±2.5

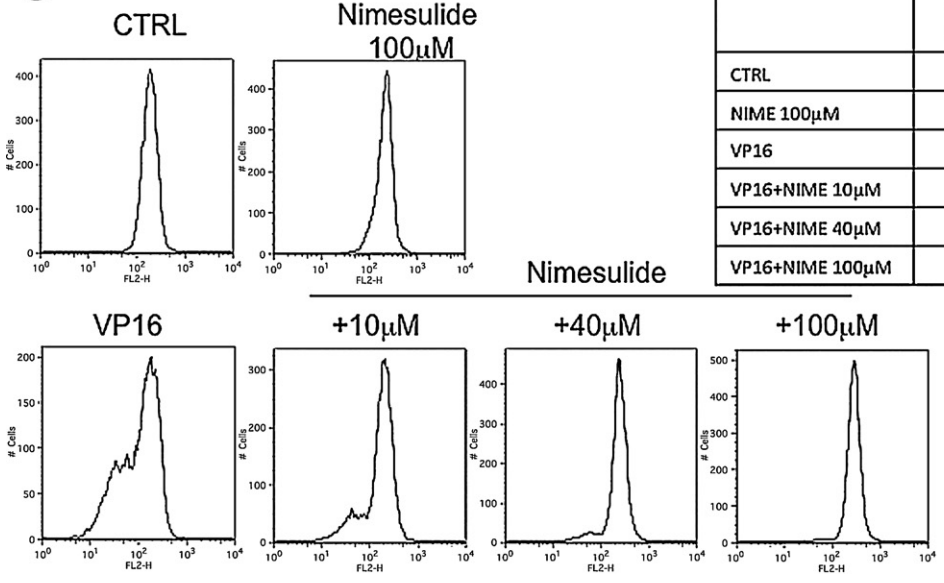


Fig. 4. COX-2 inhibitors prevent stress-induced apoptosis prior to Bax activation. Evaluation of the impact of COX-2 inhibitors on: (A) caspase-9 cleavage; (B) mitochondrial membrane potential after incubation with MitoTracker Red (50 nM; MTR) and relative quantification of the percentage of cells with high/low MTR fluorescence (right panel); (C) cytochrome *c* release from mitochondria after immunostaining of U937 cells (right panel: quantification of the percentage of the cells with low mitochondrial cytochrome *c*); (D) Bax activation by VP16 in untreated *vs.* nimesulide-treated U937 cells (100 μM nimesulide chosen as a model), as assessed by fluorescence microscopy and FACS (right panels) analysis using antibodies specific for the active form of Bax (anti-Bax 6a7) in cells counterstained with Hoechst to allow visualization of the nuclear morphology; (E) the pattern of Bax localization on U937 cells on the same samples. Mitochondria was labeled with anti-COX IV antibody and Bax with anti-Bax Δ21. One of three independent experiments with similar results is shown.

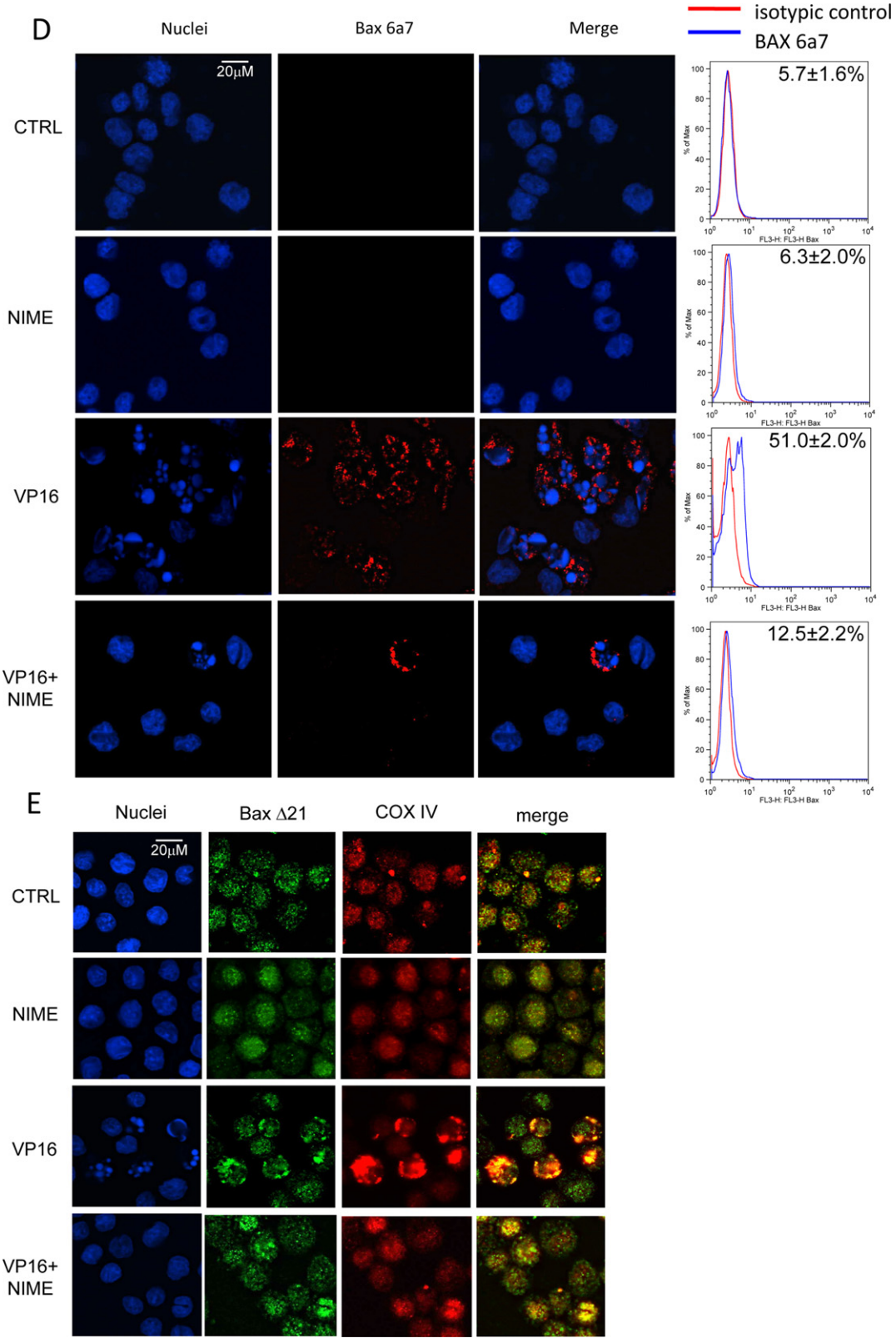


Fig. 4. (*Continued*).

extended our investigations to a panel of hematopoietic cell lines, selecting different models, heterogeneous for COX-2 protein expression levels and origins (Fig. 7A; see also Section 2). Fig. 7B documents the effects of nimesulide and celecoxib on apoptosis induced by cisplatin (an agent which efficiently impacted the viability of all the panel of cell lines tested). Nimesulide prevented apoptosis in all the cell lines, with the exception of K562 cells. Celecoxib always inhibited apoptosis.

These results confirm that anti-apoptotic potential of COX-2 inhibitors; moreover, looking at the differential levels of COX-2 protein expression (Fig. 7A), it provides further evidence that COX-2 enzyme is not implicated in this modulatory effect.

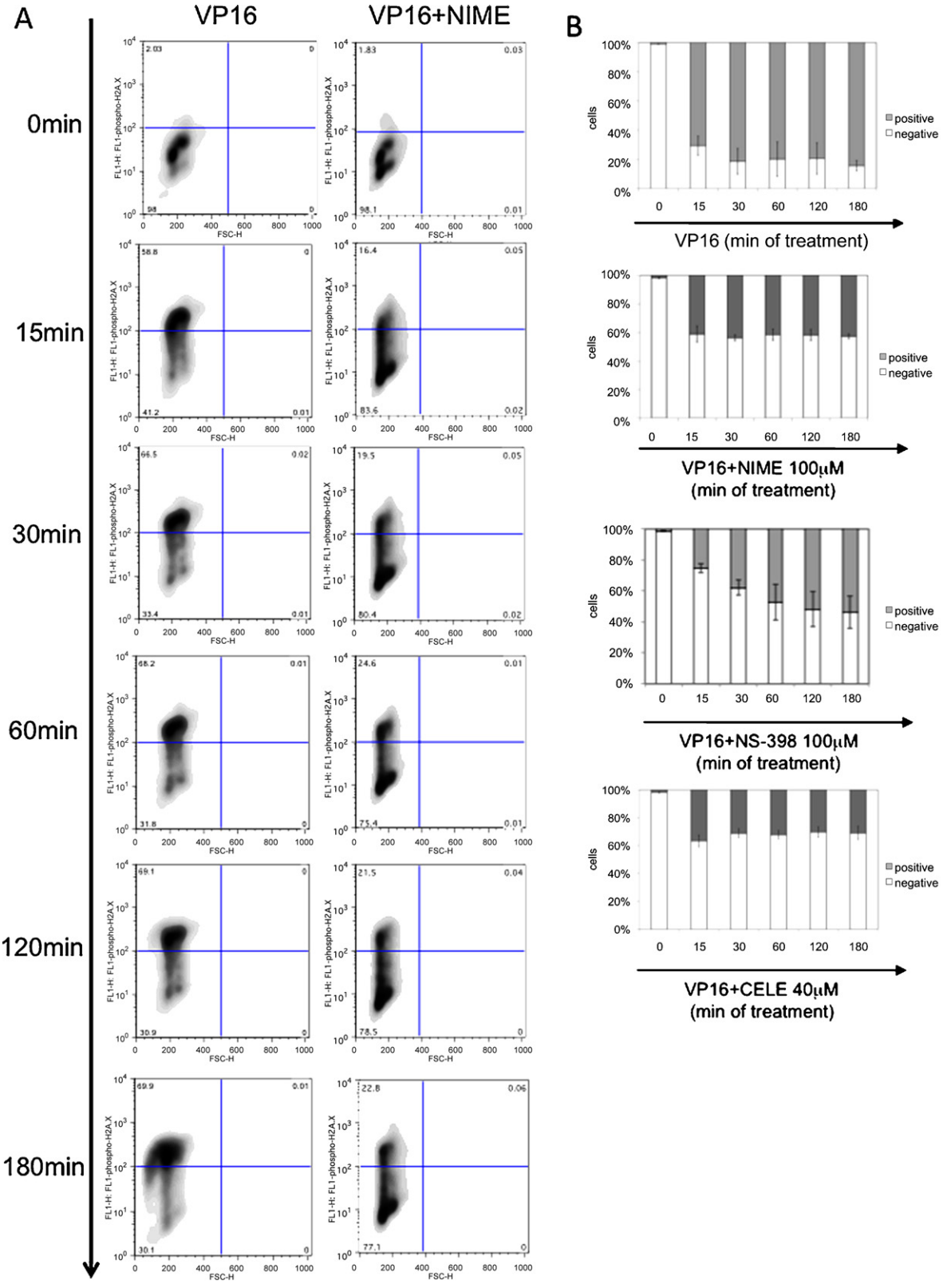


Fig. 5. COX-2 inhibitors prevent etoposide-induced DNA damage. Time-course analysis of the impact of nimesulide (100 μM), NS-398 (100 μM) and celecoxib (40 μM) on the ability of VP16 to induce DNA-damage based on the detection of the phosphorylation of the histone H2A.x (γH2A.x). (A) Typical bi-parametric analysis of anti-γH2A.x *vs.* Forward Side Scatter (FSC-H) of VP16-treated (first column) *vs.* VP16 + nimesulide-treated (VP16 + NIME) U937 cells (second column). (B) Quantification of γH2A.x-positive *vs.* negative cells in either control cells or treated with nimesulide, NS-398 or celecoxib. The results are the mean of three independent experiments ±SD.

4. Discussion

In this study, we report that three different COX-2 inhibitors (nimesulide, NS-398 and celecoxib) efficiently counteract the apoptogenic activity of a broad panel of anti-cancer agents, which are currently used in clinics. The preventive effect specifically concerns compounds that trigger the intrinsic apoptotic signaling. In contrast, COX-2 inhibitors do not exert any modulatory effect on

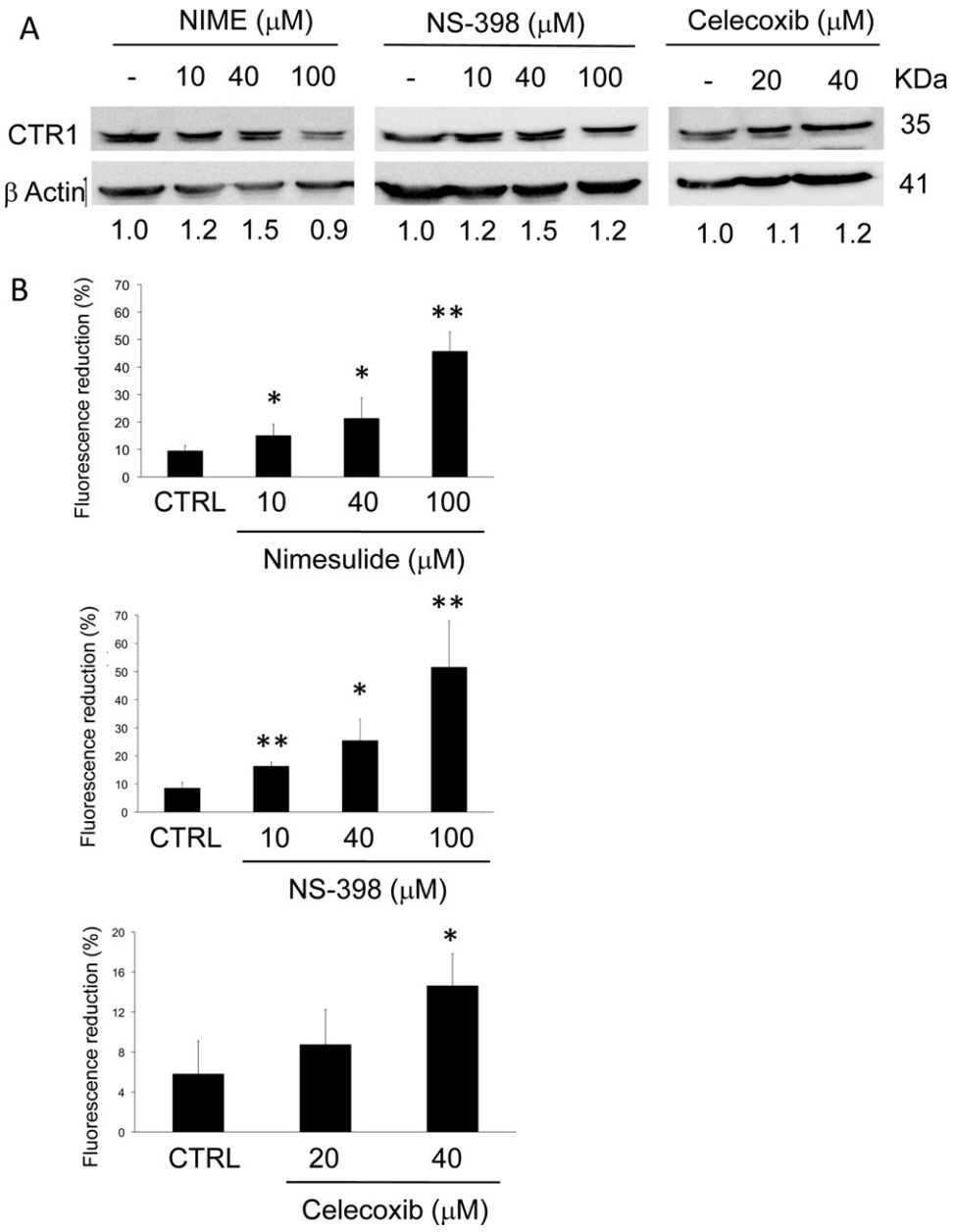


Fig. 6. Impact of COX-2 inhibitors on drug efflux and chemoresistance protein expression in U937 cells. (A) Evaluation of the impact of COX-2 inhibitors on the protein expression of the importer CTR-1. One of two independent experiments is shown. (B) Drug efflux assay based on the use of the fluorescent tool rhodamine 1,2,3 (Rh 123) in the presence/absence of the indicated concentrations of nimesulide (first row), NS-398 (second row) or celecoxib (third row). Estimation of Rh 123 fluorescence by FACS analysis immediately after Rh 123 removal ($T = 0$ h) or after 3 h of recovery ($T = 3$ h). COX-2 inhibitors were re-added during the recovery. Estimation of the percentage of the fluorescence reduction detected in untreated *vs.* treated cells (bottom panels). Analysis of expression of MDR-1 (A) and MRP-1 (B) multi-drug resistance proteins after 24 h of incubation with the reported concentrations of COX-2 inhibitors. Data correspond to three independent experiments ± SD. Significant differences when compared to control cells are: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

apoptosis induced by extracellular ligands (anti-Fas, TNFα and Trail). The intrinsic apoptotic cascade is inhibited at very early steps, prior to Bax/Bak activation. Moreover, etoposide, used as a tool to monitor the ability of chemotherapeutic agents to trigger cell damage, documents that COX-2 inhibitors strongly attenuate the generation of the apoptogenic stress.

Our results show that COX-2 inhibitors affect only the activity of stress-inducing agents. This is not the consequence of a differential modulation of the intrinsic *vs.* extrinsic apoptotic pathways. On one hand, we may only witness the prevention of the apoptotic signaling cascade without detecting any specific alterations of modulators of the intrinsic pathway. On the other hand, remarkably, the apoptogenic potential of H_2O_2, a robust oxidative agent that triggers a typical stress-induced apoptosis, exactly as found for physiological stimuli, is not affected by COX-2 inhibitors (see supplemental Fig. 6). The prevention of apoptosis is a real rescue from death and not simply a delay of apoptotic signaling. COX-2 inhibitors, indeed, prevent DNA damage induced by etoposide. This finding implies that COX-2 inhibitors act very upstream, prior to the commitment to apoptosis, because the damaging stress induced by chemotherapeutic agents (the apoptogenic stimulus thus) may be avoided.

The anti-apoptotic effect of COX-2 inhibitors reported in this study refers to 24 h of pre-treatment with the COX-2 inhibitors. We have further ascertained that the maximal anti-apoptotic effect of COX-2 inhibitors (as observed after 24 h) reaches the protection

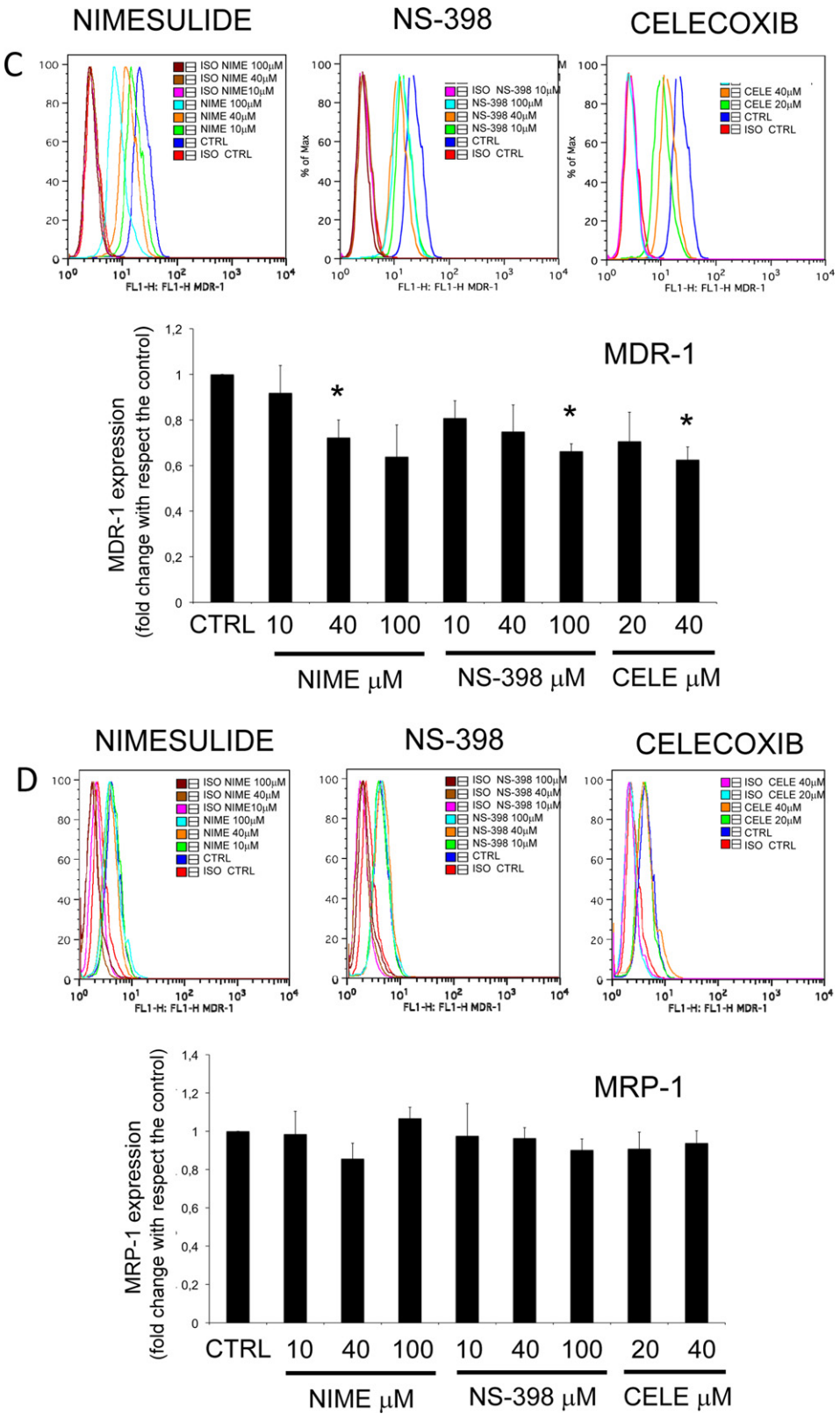


Fig. 6. (*Continued*).

plateau within 9 h of pre-treatment (supplemental Fig. 7). Cancer cells may develop different chemoresistance strategies to modulate the intracellular concentration of anti-cancer drugs [39,41]. They may up-regulate specific protein carriers, which mediate the extrusion of xenobiotics to the extracellular compartment. Conversely, they may show a decreased expression of protein importers limiting the internalization of chemotherapeutic agents. Finally, they may exacerbate specific intracellular systems relying on drug metabolizing enzymes minimizing their biological activities. Eventually these events reduce the intracellular

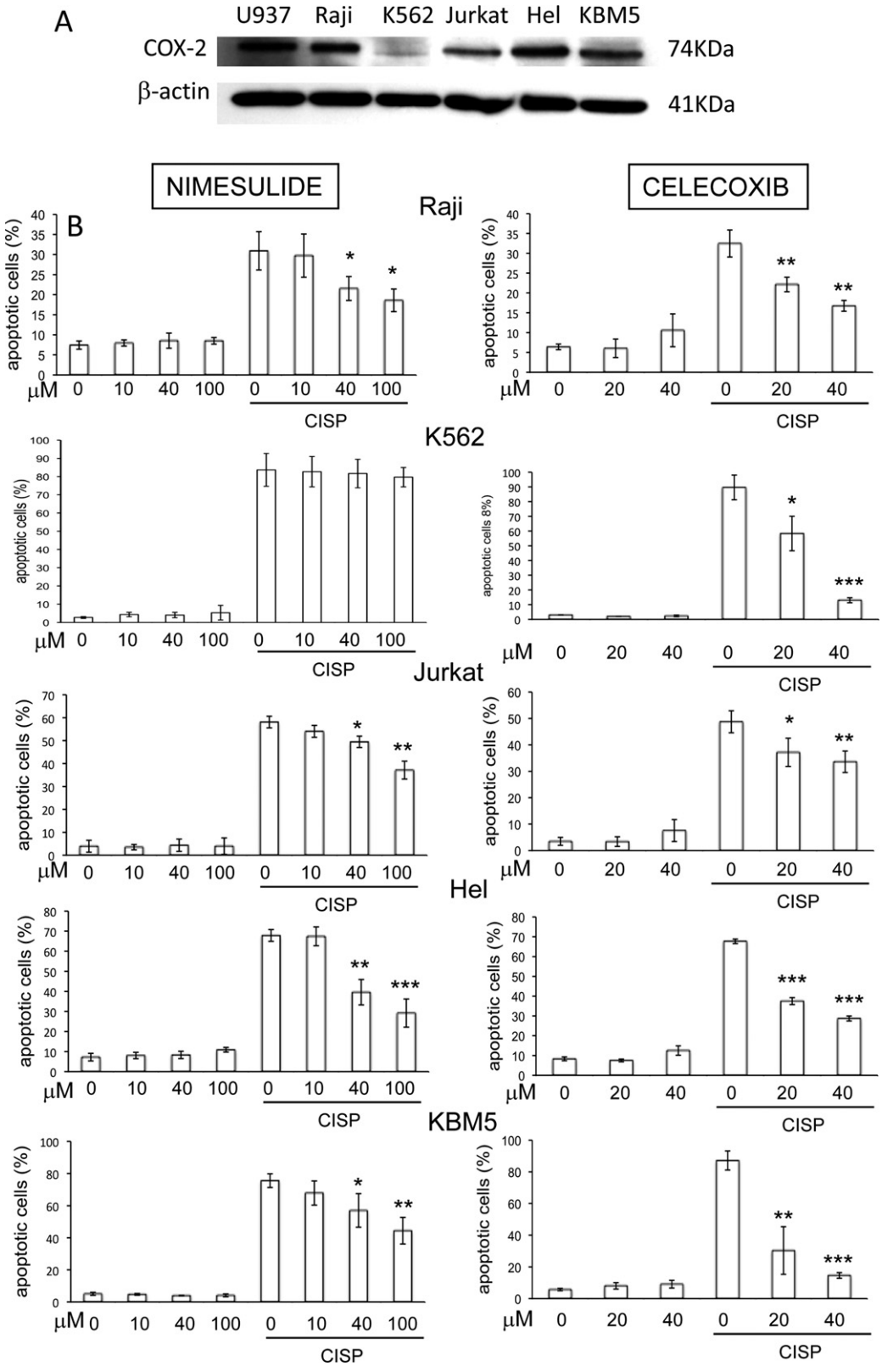


Fig. 7. COX-2 inhibitors exert anti-apoptotic effects in a broad panel of hematopoietic cancer cell lines. (A) COX-2 protein expression levels in a panel of hematopoietic cell lines (comparison with U937 cells, first band on the left) by Western blot analysis. (B) Estimation of apoptosis as assessed by analysis of the mitochondrial membrane potential loss with MTR, untreated or pre-treated 24 h with the indicated concentrations of nimesulide (first column) or celecoxib (second column). Results were confirmed by analysis of nuclear morphology after Hoechst staining (data not shown). Mean of three independent experiments ±SD. Significant differences when compared to VP16-treated cells: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

concentration of active chemotherapeutic agents below the apoptogenic threshold [39,41].

We have explored the ability of COX-2 inhibitors to modulate drug accumulation. We have found that the incubation of the cells with nimesulide and NS-398 reduces the intracellular accumulation of Rh 123, a fluorescent tool commonly used to evaluate chemoresistance due to enhanced drug efflux towards the extracellular environment [42]. However, we did not confirm the same ability for celecoxib, which very mildly affects drug efflux only at the highest concentration. Besides, when we examined the expression of the two most ubiquitously up-regulated multidrug resistance proteins in cancer cells, MDR-1 and MRP-1, we could not find any protein up-regulation, although their mRNA levels were paradoxically strongly increased, even in the case of nimesulide and NS-398. These findings do not support the hypothesis that an exacerbated phenomenon of drug extrusion may be generally responsible for the inhibition of apoptosis by COX-2 inhibitors. Similarly, initial data does not support the fact that reduced drug import may be implicated. COX-2 inhibitors appear less effective in protecting cells from apoptosis induced with puromycin, a protein synthesis inhibitor (supplemental Fig. 4). These findings suggest that the neosynthesis, rather than a down-regulation, of proteins is implicated; moreover, they indicate that the up-regulation is a reversible event. Besides, we did not observe any modulation of CTR-1 protein (importing chemotherapeutic agents including cisplatin [43]), which has been previously found up-regulated by celecoxib [21]. However, further investigations are required to exclude that other importers may be involved. Moreover, other mechanisms may also be potentially implicated. Amongst them, we may consider regulation of phases I and II drug metabolism [44]. In this context, good candidates for investigation are the superfamily of the cytochromes P450 (CYPs) [45,46] and the group of glutathione *S*-transferases (GST) [47].

COX-2 inhibitors may produce their effects dependent on or independent of the COX-2 enzyme. The evidence we collected are in favor of an off-target effect. We demonstrate here that the low basal PGE_2 production in U937 cells, our main cell model, is not modulated by the incubation with COX-2 inhibitors, even when they are used at very high concentrations; in addition, the non active analog of celecoxib, 2,5-dimethyl celecoxib [30], is similarly able to protect cells against apoptosis. The anti-apoptotic potential of COX-2 inhibitors is not limited to a specific cell type, since different hematopoietic cancer cell models appear similarly modulated. The panel of selected hematopoietic cell lines investigated is heterogeneous for biological characteristics as well as for COX-2 protein expression. The fact that COX-2 inhibitors, nevertheless, generally counteract apoptosis reinforces our evidence that the phenomenon is unrelated to COX-2 enzyme inhibition. Besides, it implies that the anti-apoptotic potential of COX-2 inhibitors is ubiquitous; however the current lack of a general target makes also impossible to predict which cells might be mostly sensitive.

A very recent study showed an ability of celecoxib to down-regulate an importer specific for cisplatin in an adherent cancer cell model [21]. Taken together, our and other findings encourage speculation that COX-2 inhibitors, in opposition to previous reports, may exert anti-apoptotic effects in a large and heterogeneous group of cancers where the exacerbation of various chemoresistance-related phenomena plays a determining role.

The mean blood concentration of nimesulide is estimated as 20 μM (6 μg/μl) after administration of 100 mg nimesulide, with peaks of 37.6 μM (11.6 μg/μl) after one week of treatment [48]. These values refer to the intake of the compound for anti-inflammatory purposes taking into account the plasma proteins binding. However, at the sites of inflammation the effective concentration is expected to be higher, as observed for other compounds normally bound by albumin, due to the lower pH [49]. In our hands, the anti-apoptotic effect of nimesulide is already significant from a minimum concentration of 10 μM (in presence of 10% FCS), thus in the range of therapeutic concentrations. Subjects exposed to chemotherapeutic protocols typically develop inflammatory conditions as a consequence of macrophage engulfment. Thus, it is conceivable that the effective concentration of these compounds in the tumor microenvironment may even be higher. Besides, different COX-2 inhibitors are currently under investigation for anti-cancer strategies. In this instance, the administration of higher concentrations is under evaluation. At the light of these considerations, the administration of these anti-inflammatory agents in association with chemotherapeutic agents should be carefully re-evaluated.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2011.06.028.

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