Potentiation of doxorubicin-induced apoptosis and differentiation by indomethacin in K-562 leukemia cells*

Ľ. ČIPÁK[1], H. PAULÍKOVÁ[2], L. NOVOTNÝ[3], M. JAROŠOVÁ[2], P. RAUKO[1]

[1]Cancer Research Institute, 833 91 Bratislava, Slovak Republic, e-mail: exonrau@savba.sk; [2]Department of Biochemistry and Microbiology, Faculty of Chemical and Food Technology, Slovak University of Technology, 812 37 Bratislava, Slovak Republic; [3]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Kuwait University, P.O.Box 24923 Safat, 13110 Kuwait

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In this study we have examined the antitumor effect of combined administrations of indomethacin (IND) with doxorubicin (DOX) on growth of K-562 leukemia cells. Although, as single drug treatment, only high concentrations of IND reduced growth (>200 μM) and induced apoptosis (>800 μM) of the K-562 cells, a synergistic effects on DOX-induced cell growth inhibition, apoptosis and differentiation were observed during the co-administration of DOX with 10 μM IND. Cells treated with this combination had elevated GSHt level compare to DOX-treated cells. Modulation of GSHt level of DOX-treated cells with Cd^{2+} ions or BSO confirmed its important role in processes of DOX-induced differentiation. Results of this study showed that IND has a positive effect on therapeutic efficacy of DOX, and could be a perspective modulator in cancer chemotherapy.

Key words: indomethacin, doxorubicin, GSH, apoptosis, differentiation, synergism

Indomethacin (IND), one of the most effective nonsteroidal anti-inflammatory drugs, is used clinically to treat inflammatory diseases, including rheumatoid arthritis and gout. It has been reported that it might directly influence proliferation of cancer cells by arresting them in G_1 phase of cell cycle and cause apoptosis [11, 12]. Some authors have showed that it potentiates the differentiation of HL-60 cells when applied in combination with small dose of retinoic acid or low levels of vitamin D3 [1, 14], or acts synergistically with cis-Pt and DOX on tumor growth inhibition [7]. Recently, ZHANG et al have demonstrated that IND sensitizes K-562 cells to VP-16 treatment and that it may induce apoptosis by down-regulated bcl-2 expression or by induction of caspase activity [18, 19].

In the present study, we have investigated the effects of IND on DOX-treated K-562 human chronic myelogenous leukemia cells. We examined the inhibitory effects of this combination on leukemia cell growth. As IND was shown to act synergistically with DOX, we also investigated its effect on DOX-induced differentiation and apoptosis. In addition, GSHt level and GST and GR enzyme activity changes were examined to look for they role in IND sensing of K-562 cells to DOX treatment.

Material and methods

Drugs. Doxorubicin (DOX) and indomethacin (IND) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The stock solutions of DOX in H_2O (4 μM) and IND in DMSO (0.5 M) were stored at –20 °C. The final concentration of DMSO in the medium was <0.2% and did not affect cell growth [3].

Cell culture and treatment. Human chronic myelogenous leukemia cells K-562 were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FCS (Grand Island Biological Co., Grand Island, NY, USA), 100 U/ml Penicillin G, 100 μg/ml streptomycin, and 2 mM L-glutamine (Sebac, Germany) at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were treated with 40 nM or 2 μM

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DOX, 1–800 μM IND, or their combinations at the beginning of the exponential growth phase for 24 h or 72 h. Cell growth and viability were assessed by direct counting of trypan blue dye-excluding cells.

Analysis on drug combination. The growth inhibition data were analyzed using Calcusyn program to determine the IC_{50} of each drug alone. The combination index (CI)-isobologram by Chou and Talalay was used to analyze the drug combinations [9]. Variable ratios of drug concentrations were used in the studies, and mutually exclusive equations were used to determine the CIs. Each CI was calculated from the mean affected fraction at each drug ratio concentration (triplicate). CI>1, CI=1, and CI<1 indicate antagonism, additive effect, or synergism, respectively.

Electrophoretic analysis of apoptotic DNA fragmentation. The untreated cells and drug-treated cells ($1x10^6$) were harvested, washed in PBS and then lysed in 100 μl of solution (10 mmol/l TRIS, 10 mmol/l EDTA, 0.5% Triton-X) supplemented with proteinase K (1 mg/ml, Serva, Germany). Samples were then incubated at 37 °C for 1 h and heated at 70 °C for 10 min. Following lysis, RNA-ase (200 μg/ml, Serva) was added and followed by repeated incubation at 37 °C for 1 h. The samples were electrophoresed at 40 V for 3 h in 2% (w/v) agarose gels (Sigma) complemented with ethidium bromide (1 μg/ml, Sigma) [5]. Separated DNA fragments (DNA ladders) were visualized using UV transilluminator (254 nm, Ultra-Lum Electronic UV Transilluminator, USA).

Differentiation assay. The percentage of hemoglobin-producing K-562 cells was determined after 72 h treatment by benzidine staining method [10]. Briefly, 5 μl of 30% H_2O_2 was added to 1 ml of benzidine dihydrochloride (Sigma) solution (2 mg/ml in 0.9% NaCl plus 0.5% acetic acid). Equal volumes of the benzidine solution and the cell suspension were mixed and incubated for 10 min at room temperature (RT) and the cells were scored in a hemocytometer as benzidine-positive (blue) or benzidine-negative (yellow).

Determination of total glutathione (GSHt) content. Total cellular glutathione (GSHt) was quantified using glutathione reductase-DTNB recycling assay modified for 96-well microtiter plates [15]. Cells were washed with PBS (4 °C) and lyzed by freezing and thawing in 150 μl of 10 mM HCl. Proteins were precipitated by adding 30 μl of 6.5% 5-Sulfosalicylic acid. After 10 min tubes were centrifuged (15 min, 2000 g, 4 °C) and supernatants were stored at –20 °C. 40 μl of the supernatant was mixed with 80 μl of PBS (200 mM, pH 7.5, supplemented with 2 mM EDTA) and 20 μl of 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB) (0.4 mM) was added. The plate was kept 5 min at RT. Following incubation, 10 μl of NADPH (0.2 mM) and 20 μl of Glutathione reductase (GR) (8.5 IU/ml) were added. The increase of absorbance at 410 nm was monitored for 10 min and used to determine the GSHt content in the sample by comparison to a reference curve generated with known amounts of GSH. GSHt levels were normalized for protein content (nmol/mg).

GST activity assay. Glutathione S-transferase (GST) activity was determined according to the method of HABIG and JAKOBY [6]. Cells were washed with PBS (4 °C) and lyzed by freezing and thawing in 150 μl of H_2O. Tubes were centrifuged for 15 min (10 000 g, 4 °C). GST activity of cell lysate was determined by measuring activity towards the GST substrate 1-chloro-2,4-dinitrobenzene (CDNB) by following the rate of increase of absorbance at 340 nm. The assay mixture contained 0.1 M PBS (pH 6.5), 0.4 mM GSH, and 0.2 mM CDNB and 50–100 μl of cell lysate. The ε_{340} = of DNPSG is 9.6 $mM^{-1}.cm^{-1}$. GST activity was expressed as nmol of CDNB conjugated with GSH/min/mg protein.

GR activity assay. Glutathione reductase activity was determined by monitoring the oxidation of NADPH at 340 nm [2]. The assay mixture contained 0.1 M PBS (pH 7.15), 1 mM EDTA, 50 μM NADPH, 1 mM oxidized glutathione (GSSG) and 50–100 μl of cell lysate. GR activity was calculated as nmol of NADPH oxidized/min/mg protein using molar extinction coefficient ε_{340} = 6.22 $mM^{-1}.cm^{-1}$.

Results

Indomethacin inhibits proliferation and induces apoptosis. Previous studies have shown that IND inhibits proliferation of various cancer cell lines [4, 11, 17]. Here, we tested its effects on proliferation of K-562 leukemia cells. We found that IND induced decreasing of cell concentration by time-dependent inhibition of cell proliferation (Fig. 1). Values of IC_{50} for 24 h and 72 h treatment were 245.2 and 61.7 μM, respectively. To look if IND induces apoptosis, we treated K-562 cells with various concentrations of IND (10–800 μM). We found that apoptosis was sufficiently induced when cells were treated with 800 μM IND for 24 h. Continued treatment (48 h) with 400 μM IND was ineffective in inducing apoptosis (Fig. 2A). Unfortunately, 800 μM IND completely inhibited cell proliferation, and decreased their viability to 84.7%.

Indomethacin acts synergistically with DOX and potentiates DOX-induced differentiation and apoptosis. Despite of the high concentrations of IND used for inhibition of proliferation and induction of apoptosis, next we looked if IND affects the efficacy of DOX. We found that non-cytotoxic concentrations of IND (1–10 μM) in combination with DOX acted synergistically. Combination drug analysis have shown (Tab. 1) that combinations of 40 nM DOX and 1–10 μM IND for 72 h led to synergism in DOX-inhibitory activity on cell proliferation (CIs<1). Additionaly, 10 μM IND increased the differentiation of K-562 cells when combined with 40 nM DOX (33.6 $\pm$ 3.3%), compare to differentiation of cells treated with DOX alone (19.4 $\pm$ 2.8%) (Fig. 3). This

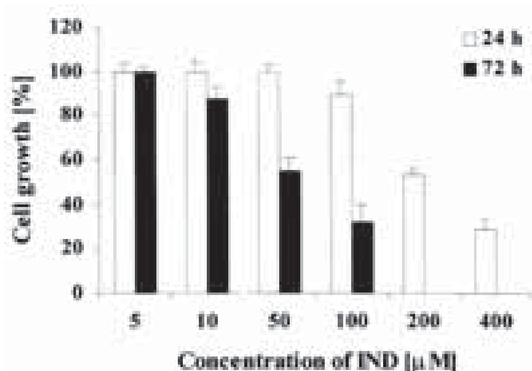

Figure 1. Inhibitory effect of IND on proliferation of K-562 cells. Cells were exposed to IND for 24 or 72 h and cell growth was determined by trypan blue staining. The columns represent mean values ±S.D. obtained from three independent experiments.

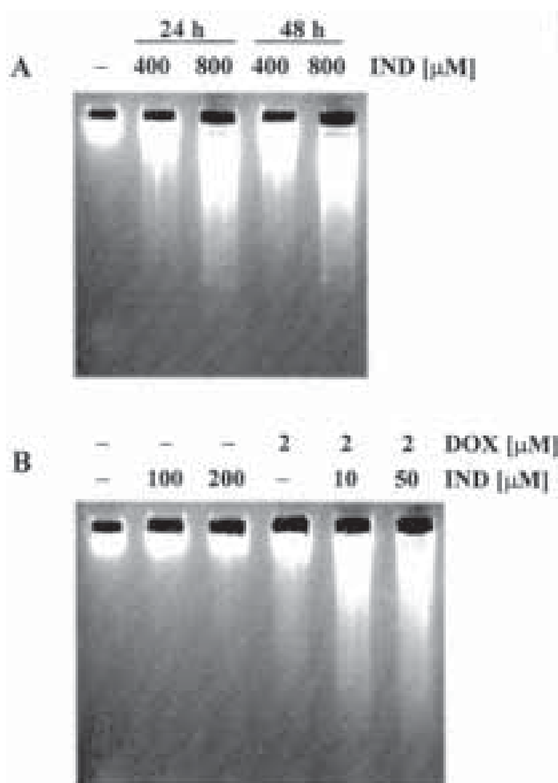

Figure 2. (A) Apoptotic DNA fragmentation of K-562 cells exposed to IND for 24 and 48 h. (B) Detection of apoptotic DNA fragmentation in K-562 cells treated with IND, DOX or their combinations for 24 h. These figures are representative of three separate experiments.

concentration of IND also sensitized the cells to DOX-induced apoptosis. We detected enhanced apoptotic DNA fragmentation when combined 10 μM IND with 2 μM DOX (Fig. 2B).

Indomethacin increases the GSHt level and changes the activities of GSH-related enzymes. One of the mechanism of action of DOX is the generation of ROS that may change the redox environment of the cell, which tightly regulate apoptosis and differentiation [13]. We therefore looked how IND influence the ratio of GSH/GSSG, the main redox marker of the cells. We found that 10 μM IND alone increased the content of GSHt significantly after 72 h treatment. The GSHt level was increased significantly in cells treated with 10 μM IND and 40 nM DOX for 24 h, compared to the cells treated with 40 nM DOX. The same level of GSHt was detected after 72 h of combinational treatment, despite of the fact that in cells treated with DOX alone for 72 h the level of GSHt declined when compared to the control (Fig. 4A). Additionaly, in cells treated with 10 μM IND and 40 nM DOX for 72 h the activity of GST (enzyme involved in GSH metabolism) was increased (238% of control) (Fig. 4B), and significantly increased level of GSSG in thus treated cells (5 μmol GSSG/g prot.), as well as decreased activity of GR (60% of control) were detected (Fig. 4C).

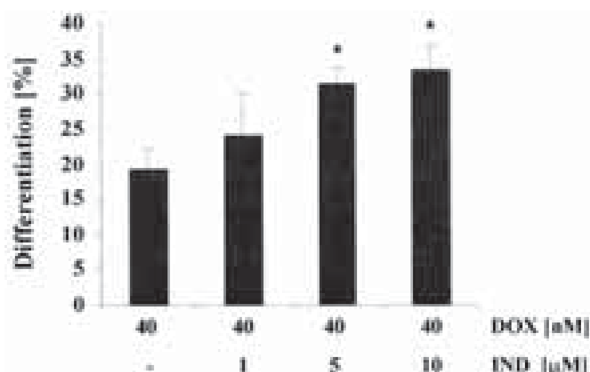

Figure 3. Effects of DOX and IND on differentiation of K-562 cells. Exponentially growing cells were treated as indicated, and percentage of differentiation was determined as described in Materials and methods. Data are the mean ±S.D. of three independent experiments.
$^{*}p<0.05$ as compared with DOX-treated cells.

Table 1. Combined effects of DOX and IND on K-562 cell proliferation (72 h).

Agents	CI[a] value
40 nM DOX + 1 μM IND	0.926
40 nM DOX + 5 μM IND	0.759
40 nM DOX + 10 μM IND	0.715

[a]CI was calculated as described in Materials and methods.

Modulation of GSHt level by BSO and Cd^{2+} ions confirms the connections between indomethacin-induced changes in GSHt level and its synergistical activity on DOX-induced differentiation. To confirm that GSHt level changes are involved in modulation of DOX-induced differentiation, we used BSO and Cd^{2+} ions to modulate the GSHt level. We

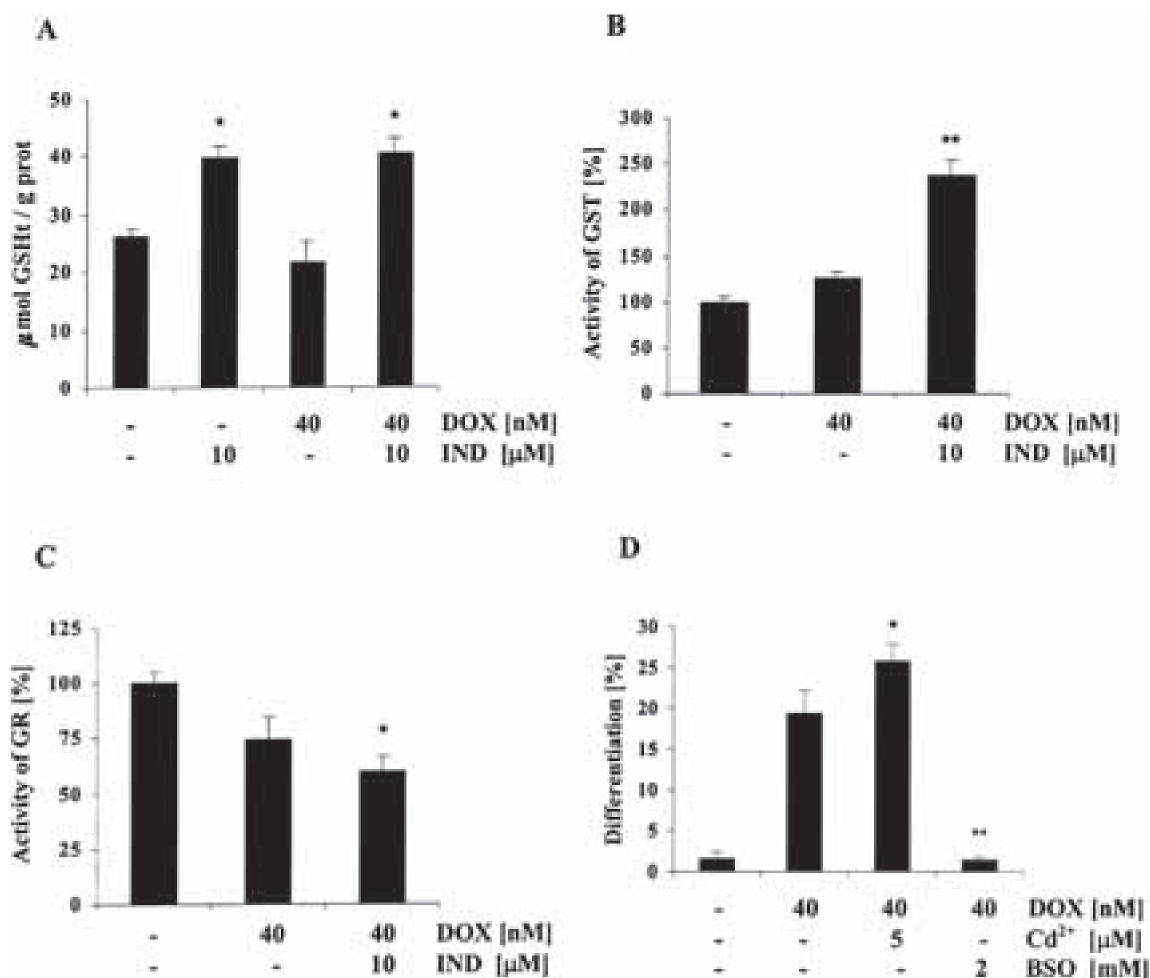

Figure 4. Effects of DOX and IND on (A) GSHt level, (B) activity of GST and (C) activity of GR. Cells were exposed to tested drugs as indicated, and the changes in GSHt level, and activities of GST and GR enzymes were determined as described in Materials and methods. (D) Effect of Cd^{2+} ions and BSO on DOX-induced differentiation. Cells were treated with DOX in combination with GSH modulators and percentage of differentiation was determined as described in Materials and methods. The columns represent mean values $\pm$S.D. obtained from three independent experiments.
$^{*}p<0.05$, $^{**}p<0.01$ as compared with non-treated cells (A,B,C) or DOX-treated cells (D), respectively.

found that differentiation of cells treated with 40 nM DOX and 5 μM Cd^{2+} ions (they increase GSHt level) was 133% of DOX-treated cells. On the other hand, in cells treated with combination of 40 nM DOX and 2 mM BSO (inhibitor of GSH synthesis) the differentiation of cells was completely abolished (Fig. 4D).

Discussion

In the present work, we have demonstrated that IND, a nonsteroidal anti-inflamatory drug, is capable to sensitize the K-562 leukemia cells to DOX treatment. Despite of the fact that we have used high concetrations (>200 μM) of IND to approach its antiproliferating effect (see Fig. 1) or to induce apoptosis (see Fig. 2A), we found that low concentrations (1–10 μM) of IND markedly potentiated the DOX-induced K-562 cells apoptosis and differentiation. When DOX was applied to K-562 cells alone at 2 μM concentration for 24–48 h apoptotic changes were not detected. Adding of 10 μM IND for co-incubation led to evident apoptotic DNA fragmentation (see Fig. 2B). These results suggest that IND has a synergistic effect on DOX-induced apoptosis of K-562 cells. Additionally, the same concentration of IND increased the percentage of cells that undergo differentiation after treatment with DOX (Fig. 3). Our observation supported by ZHANG et al, who demonstrated that IND sensitize K-562 cells to VP-16 treatment [18], suggests that IND may enhance the chemotherapeutic sensitivity of K-562 cells, and alleviate the toxic-side action at higher concentrations of these drugs.

We thus hypothesize that in the case of DOX-treated K-562 cells when ROS are produced [8], IND may modulate the redox state of the glutathione couple of the cells. It is well known that changes in redox state of the cells are crucial for decision of cells to proliferate, to die or to differenti-

ate [13]. To verify our hypothesis, next we looked for changes in GSHt level of cells treated with combinations of these drugs. As we presented in Figure 4A, GSHt level of DOX-treated K-562 cells is slightly increased after 24 h treatment. Combination of IND with DOX induced significant elevation of GSHt level when compared to DOX-treated cells. In contrast to DOX-treated cells, which decline GSHt level after 72 h, the cells treated with combination of IND and DOX have unchanged GSHt level, when compared to 24 h DOX/IND-treated cells. Additionally, the level of GSSG was significantly increased in cells treated with DOX and IND for 72 h due to decreased activity of GR (Fig. 4C). These observations showed on the correlation between the redox state of the cells and potentiation of DOX-induced differentiation. The important role of redox state in processe of DOX-induced differentiation was more evident when we used modulators of GSH level (Cd^{2+} ions and BSO) (Fig. 4D), which elevate or decrease the GSH level [16].

In summary, this work indicates that IND has antiproliferative and apoptosis-inducing effects on K-562 cells. IND has synergic effect with DOX in antileukemic activity due to potentiation of DOX-induced differentiation and apoptosis. Although the IND concentrations on inducing K-562 cell apoptosis are relatively high (800 μM), the concentrations for synergic modulation of DOX-induced differentiation and apoptosis is relatively low (~10 μM).

In conclusion, our data show that IND, although therapeutically ineffective in single treatment of K-562 cells, can be useful modulator of therapeutic efficacy when coupled with appropriate chemotherapeutics, e.g. DOX. Elucidation of the precise molecular mechanisms of action of this drug combination on the level of apoptotic and differentiation pathway regulations, is the next target of our research efforts.

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