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5-Aza-2′ -deoxycytidine and paclitaxel inhibit inducible nitric oxide synthase activation in fibrosarcoma cells

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Abstract

Given the important role of gaseous free radical nitric oxide (NO) in tumor cell biology, we investigated the ability of the anti-cancer drugs 5-Aza-2′-deoxycytidine (ADC) and paclitaxel to modulate NO production in mouse L929 fibrosarcoma cells. Both drugs reduced IFN-γ-stimulated NO release in cultures of L929 and primary fibroblasts, but not in mouse peritoneal macrophages. The inhibitory effect was due to the reduced expression of inducible NO synthase (iNOS), the enzyme responsible for cytokine-induced intracellular NO synthesis, as both agents markedly suppressed the interferon-gamma (IFN-γ)-triggered increase in iNOS concentration in L929 cells. In addition, ADC and paclitaxel prevented the IFN-γ-triggered activation of p44/p42 mitogen-activated protein (MAP) kinase in L929 fibroblasts, suggesting a possible mechanism for the observed inhibition of iNOS expression. These results might have important implications for the therapeutic effect of ADC and paclitaxel, since their inhibitory action on NO release partly neutralized the NO-dependent toxicity of IFN-γ on L929 fibrosarcoma cells.

Keywords: 5-Aza-2′ -deoxycytidine; Paclitaxel; Nitric oxide (NO); iNOS (nitric oxide (NO) synthase, inducible); L929; Fibrosarcoma

1. Introduction

5-Aza-2′ -deoxycytidine (ADC) and paclitaxel (also known as taxol) are anti-cancer drugs with different tumoricidal mechanisms. As a nucleoside analogue, ADC incorporates into DNA and exerts direct cytotoxic and antiproliferative effects on tumor cells (Christman, 2002). These effects are mainly dependent on interference with DNA reparative machinery and inhibition of de novo thymidine synthesis, but also on activation of pro-apoptotic intracellular signalization (Christman, 2002; Karpf et al., 2001). In addition, at dose levels, low enough to avoid triggering cell death, incorporation of ADC into DNA leads to a rapid loss of DNA methyltransferase activity (Christman, 2002). Since tumor cells have an altered pattern of methylation of tumor-suppressor genes and oncogene regulatory sequences compared with those of normal cells (Christman, 2002), ADC-imposed inhibition of DNA methylation could contribute to the reversal of this altered gene regulation back to a normal state.

Paclitaxel is a complex diterpene isolated from the bark of the Pacific yew tree. By stabilizing the microtubules against depolymerization, Paclitaxel blocks the progression of cell division through metaphase (Schiff et al., 1979; Manfredi et al., 1982; Rowinsky et al., 1988) and efficiently suppresses tumor cell proliferation. In addition, paclitaxel exerts lipopolysaccharide-mimetic properties, including stimulation of nitric oxide (NO) and tumor necrosis factor-alpha (TNF-α) production by macrophages (Ding et al., 1990; Byrd-Leifer et al., 2001). Since macrophage-mediated elimination of transformed cells depends on both NO and TNF-α, stimulation of their release contributes to the anti-tumor effect of paclitaxel in vitro (Manthey et al., 1994).

Nitric oxide is a highly reactive free radical molecule that exerts potent biological actions in mammals, including neurotransmission, vasodilatation and immunity (MacMicking et al., 1997). The immune properties of NO have been mostly attributed to NO produced by the inducible isoform of NO synthase (iNOS) in immune cells, such as macrophages, as well as in various cell types that contribute to

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regulation of the immune response, such as endothelial cells or fibroblasts (MacMicking et al., 1997; Bogdan, 2001). NO and some of its biological derivatives are potent cytotoxic molecules, and have diffusional properties necessary for the elimination and extracellular destruction of invading cells, including tumors (MacMicking et al., 1997). However, NO has mutagenic and carcinogenic properties, and tumor cell-derived NO can promote tumor growth and metastasis through vasodilatation and stimulation of angiogenesis (Fukumura and Jain, 1998; Lala and Orucevic, 1998; Bogdan, 2001).

Given the importance of NO for tumor cell biology, we analyzed the influence of the anti-tumor drugs ADC and paclitaxel on NO production in mouse fibrosarcoma L929 cells and mouse macrophages.

2. Materials and methods

2.1. Reagents and cell cultures

All the chemicals used in the experiments were from Sigma (St. Louis, USA), unless specifically stated. The murine fibrosarcoma cell line L929 was obtained from the European Collection of Animal Cell Cultures (Salisbury, UK) and grown in HEPES-buffered RPMI 1640 medium supplemented with 5% fetal calf serum, glutamate and antibiotics (culture medium) at 37 °C in a humidified atmosphere with 5% CO_2. Plastic-adherent fibroblast-like cells derived from spleen, and endothelial cell cultures prepared from the hearts of 5-day-old CBA/H mice (animal facility of Institute for Biological Research, Belgrade, Yugoslavia) were used as previously described (Kasten, 1972; Pechold et al., 1997). Resident macrophages were obtained from CBA/H mice by peritoneal lavage with cold phosphate buffer saline and selected by adherence to plastic (Trajkovic et al., 2000). Cells were seeded in 96-well flat-bottom plates (200 μl final volume) in the following numbers (per well): L929 cells—6×10^4, macrophages—1×10^5, fibroblasts and endothelial cells—4×10^4. Cells were stimulated to produce NO with 250 U/ml of interferon-gamma (IFN-γ) (Holland Biotechnology, Leiden, Holland) in the presence of different agents, as indicated in the figure legends.

2.2. Nitrite measurement

Nitrite accumulation, an indicator of NO production, was measured in cell culture supernatants using the Griess reagent (Green et al., 1982). Briefly, 50-μl samples of culture supernatants were incubated for 10 min at room temperature with an equal volume of Griess reagent (1% sulfanilamide in 5% H_3PO_4 + 0.1% naphthylethylenediamine dihydrochloride). The absorbance at 570 nm was determined in a microplate reader. The nitrite concentration (μM) was calculated from a $NaNO_2$ standard curve.

2.3. Cell viability assay

Cell respiration, as an indicator of cell viability, was assessed by the mitochondrial-dependent reduction of 3-[4,5-Dimethylthiayol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) to formazan (Mosmann, 1983). MTT solution was added to cell cultures in a final concentration of 0.5 mg/ml, and cells were incubated for an additional hour. Thereafter, the solution was removed and cells were lysed in Dimethyl sulfoxide. The conversion of MTT to formazan by metabolically viable cells was monitored with an automated microplate reader at 570 nm.

2.4. Cell-based enzyme-linked immunosorbent assay (ELISA)

The relative amounts of iNOS protein and phosphorylated form of p44/42 mitogen-activated protein kinase (MAPK) were determined according to original protocol for the cell-based ELISA (Versteeg et al., 2000). Rabbit anti-mouse iNOS (dilution 1/10 000) or rabbit anti-mouse phospho-p44/42 MAPK (1/500; Santa Cruz Biotechnology, Santa Cruz, CA) were used as primary antibodies, and Horseradish pereoxidase-conjugated goat anti-rabbit Ig (H + L) (1/1000; US Biochemical, Cleveland, OH) was used as secondary antibody.

2.5. Statistical analysis

The data from representative of at least three independent experiments with similar results are presented as means ± S.D. of triplicate measurements. The significance of the differences between various treatments was determined by one-way analysis of variance (ANOVA), followed by Student–Newman–Keuls test. Value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. ADC and paclitaxel inhibit NO production in L929 cells, but not macrophages

In contrast to unstimulated cultures, L929 cells produced large amounts of NO upon activation with IFN-γ (Fig. 1A,B). While ADC and paclitaxel did not affect the low basal NO production in L929 cells, both drugs potently inhibited IFN-γ-induced NO release from L929 cells, in a dose- and time-dependent manner (Fig. 1A,B). Even at the highest concentrations applied, neither ADC nor paclitaxel reduced the viability of L929 cells stimulated with IFN-γ, (data not shown), thus excluding cytotoxicity as a cause for the observed inhibition of NO release. Paclitaxel and ADC also downregulated IFN-γ-induced NO production in primary mouse fibroblasts (Fig. 1E,F), thus excluding the possibility that the drugs selectively affected NO synthesis

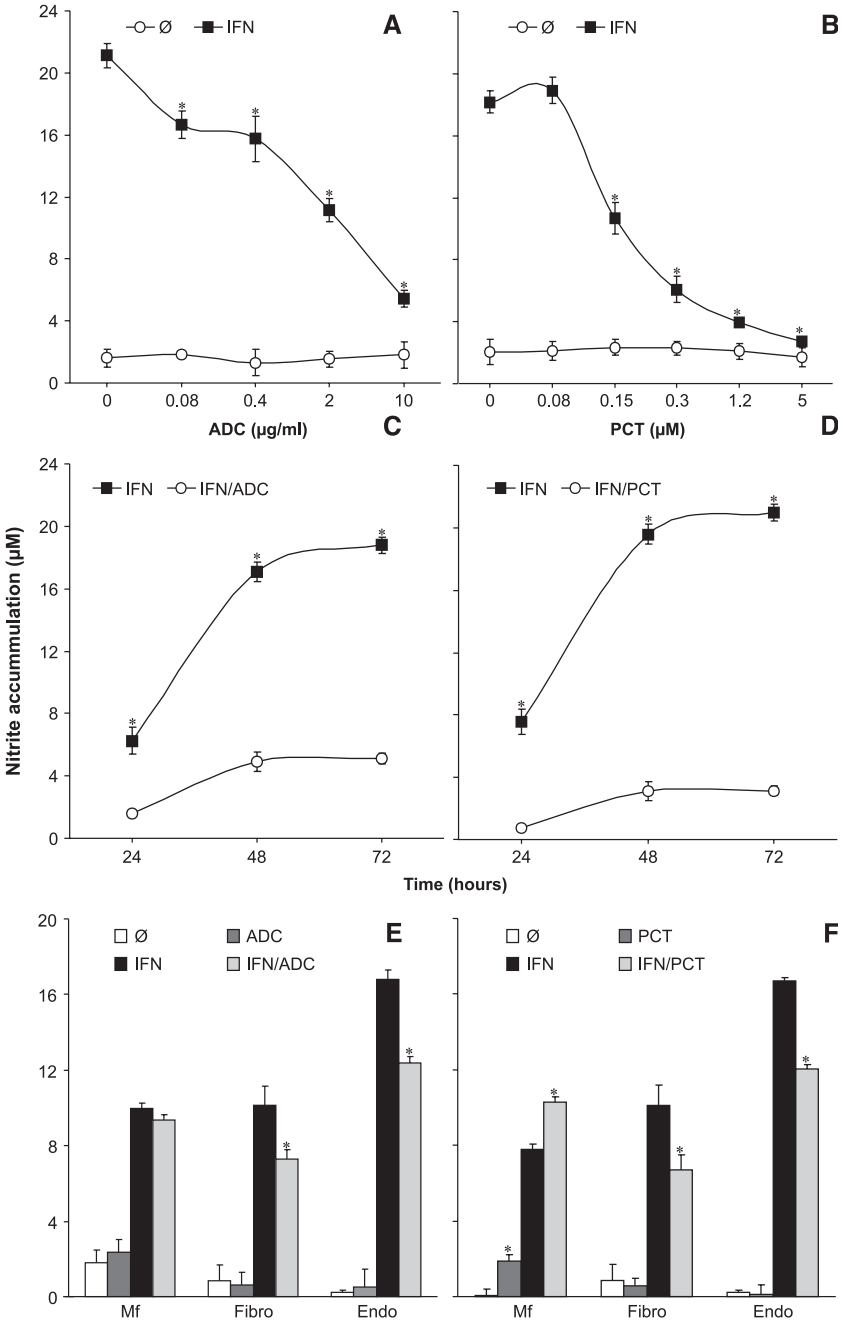

Fig. 1. The effect of ADC and paclitaxel on NO production in various mouse cell types. (A, B) L929 cells were incubated for 48 h with various concentrations of ADC or paclitaxel (PCT), in the absence or presence of IFN-γ. (C, D) L929 cells were cultivated in the absence or presence of 5 μg/ml ADC or 2.5 μM paclitaxel and/or IFN-γ for various periods of time. (E, F) Macrophages, fibroblasts and endothelial cells were cultivated in the absence or presence of 5 μg/ml ADC or 2.5 μM paclitaxel (PCT) and/or IFN-γ for 48 h. (A–F) At the end of incubation, nitrite accumulation was measured in cell culture supernatants (*$P<0.05$ refers to cultures without ADC or paclitaxel).

in tumor cells. Similarly, both drugs inhibited NO release from primary mouse endothelial cells (Fig. 1E,F). However, ADC did not affect NO release from either unstimulated or IFN-γ-stimulated mouse macrophages (Fig. 1E), while paclitaxel stimulated both basal and IFN-γ-imposed NO production in mouse macrophages (Fig. 1F). Collectively, these data indicate that ADC and paclitaxel can influence NO production in a cell-specific manner.

3.2. ADC and paclitaxel inhibit iNOS protein expression in L929 cells

The control of iNOS-mediated NO production involves both transcriptional and post-transcriptional mechanisms, the latter including modulation of the catalytic activity of iNOS enzyme. We therefore sought to determine the level at which the iNOS-mediated NO production in L929 cells was

impaired by ADC and paclitaxel. To this end, L929 fibroblasts were pretreated with IFN-γ for 24 h to induce iNOS, and any further induction was prevented by blocking transcription or translation with actinomycin D or cycloheximide, respectively. Under these conditions, any reduction of NO production would presumably stem from direct interference with iNOS-mediated oxidation of L-arginine. However, blockade of transcription or translation completely abolished both ADC and paclitaxel-mediated inhibition of NO production in L929 cells (Fig. 2A,B). Thus, it appears that iNOS expression, rather than its catalytic activity, was a target for these drugs. This assumption was confirmed by the results of a cell-based ELISA specific for iNOS protein, as both drugs markedly downregulated the IFN-γ-triggered accumulation of iNOS in L929 cells (Fig. 2C).

3.3. Mechanisms of ADC and paclitaxel influence on NO production in L929 cells

We next tried to elucidate the mechanisms responsible for the observed inhibitory action of ADC and paclitaxel on iNOS-mediated NO production in L929 cells. As MAPK p44/42 signaling is profoundly involved in the induction of iNOS gene expression in various cell types, including fibroblasts (Wang, 1999; Jankovic, 2000), we investigated the influence of ADC and paclitaxel on p44/42 activation in L929 fibrosarcoma cells. Treatment of L929 cells with IFN-γ caused the activation of p44/42 MAPK, as judged by the increased intracellular concentration of the active phosphorylated form of the kinase (Fig. 3A). Both ADC and paclitaxel effectively prevented this effect of IFN-γ, thus indicating that their influence on NO synthesis might be, at least partly, accomplished through interference with p44/42 MAPK signaling. To get a further insight into the action of ADC and paclitaxel, we investigated how drugs that mimic their intracellular action affect NO production in L929 cells. To this end, we used the demethylating agent L-ethionine (Garcia-Trevijano et al., 2000) and a drug that disrupts microtubule homeostasis by causing their depolymerization-colchicine. Without reducing cell viability (data not shown), both L-ethionine and colchicine markedly inhibited IFN-γ-triggered NO production in L929 cells in a dose-dependent manner (Fig. 3B), thus implying a demethylation-dependent and a microtubule-dependent action of ADC and paclitaxel, respectively.

3.4. ADC and paclitaxel partly rescue L929 cells from NO-dependent autotoxicity

Finally, we explored the possible relevance to tumor cell survival of the observed effects of ADC and paclitaxel. NO produced by tumor cells exerts toxic effects in an autocrine and a paracrine fashion (Samardzic et al., 2000; Jankovic et al., 2000; Miljkovic et al., 2002). Accordingly, in our hands,

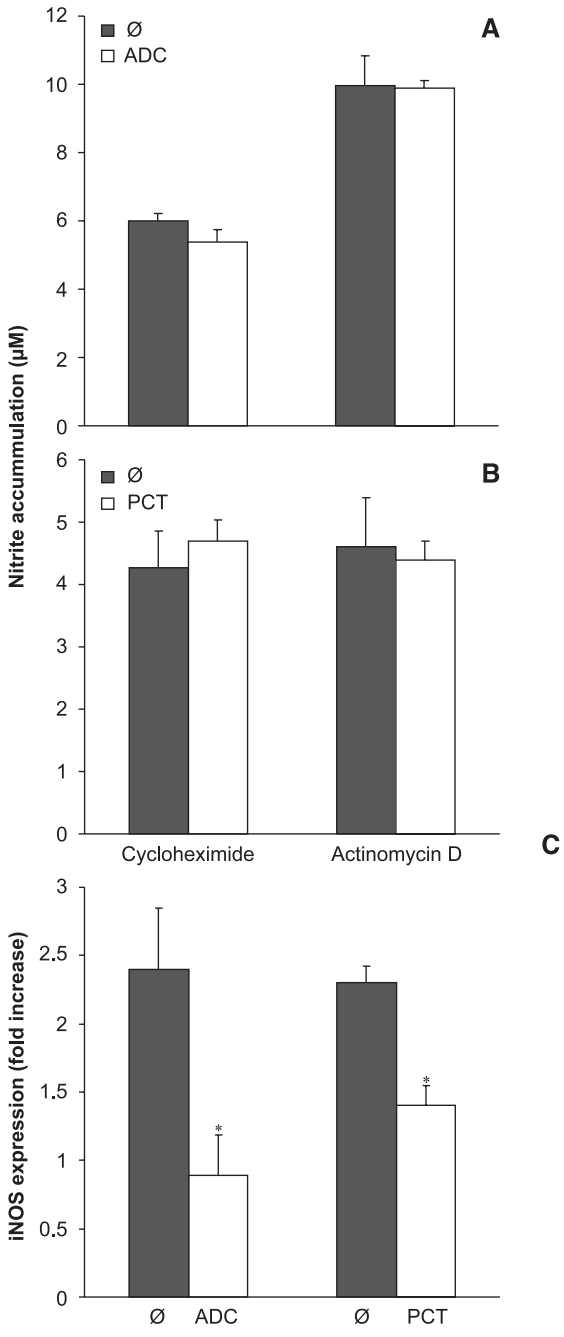

Fig. 2. Downregulation of iNOS protein expression in L929 cells by ADC and paclitaxel. (A, B) L929 cells were incubated with IFN-γ for 24 h to induce iNOS, thoroughly washed and cultivated for an additional 48 h in fresh medium containing 5 μg/ml ADC (A) or 2.5 μM paclitaxel (PCT) (B) with or without 5 μg/ml cycloheximide or 5 μg/ml actinomycin D. (C) L929 cells were stimulated with IFN-γ in the absence or presence of 5 μg/ml ADC or 2.5 μM paclitaxel (PCT) for 24 h. Subsequently, cells were fixed and a cell-based ELISA with iNOS-specific antibodies was performed as described in Materials and methods. The results are presented as fold increase relative to cell cultures without IFN-γ stimulation (absorbance value 0.23 ± 0.06).

IFN-γ-induced NO production in L929 cells coincided with a significant suppression of cellular respiration, as assessed by MTT assay (Fig. 4A). This was a consequence of NO-

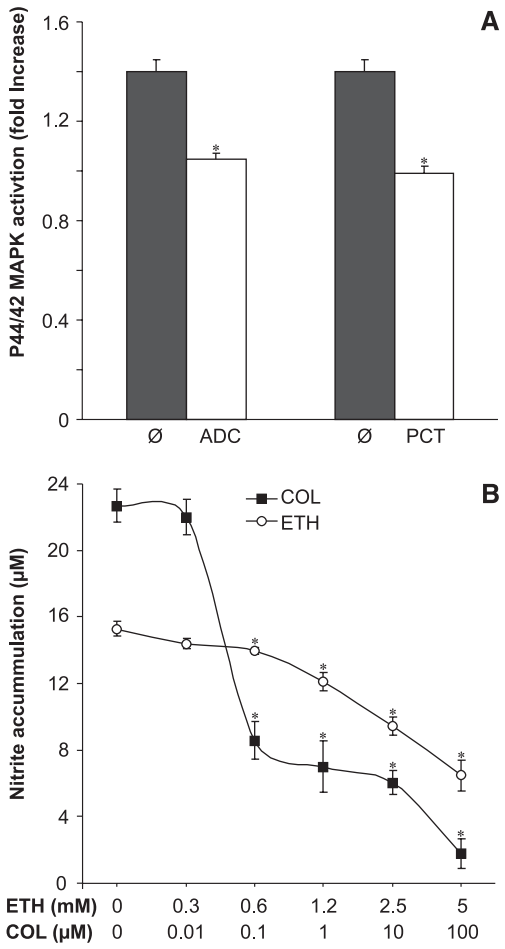

Fig. 3. Mechanisms responsible for the ADC and paclitaxel-mediated inhibition of NO synthesis. (A) L929 cells were incubated with IFN-γ in the presence or absence of 5 μg/ml ADC or 2.5 μM paclitaxel (PCT) for 30 min. Afterwards, cells were fixed and a cell-based ELISA with phospho-p44/42 MAPK-specific antibodies was performed. The results are presented as fold increase relative to cell cultures without IFN-γ stimulation (absorbance value 0.253 ± 0.011). (B) L929 cells were incubated with IFN-γ, in the absence or presence of various concentrations of L-ethionine (ETH) or colchicine (COL). Nitrite accumulation in cell culture supernatants was measured after 48 h (*$P < 0.05$ refers to cultures without ADC or paclitaxel). Nitrite accumulation in the absence of IFN-γ was < 2 μM.

mediated cytotoxicity, as the selective iNOS inhibitor aminoguanidine (Misko et al., 1993) prevented IFN-γ-induced NO production and restored cell respiration in L929 cultures (Fig. 4A). We next investigated whether the observed ADC and paclitaxel-mediated inhibition of NO release might affect the tumoricidal action of NO. When applied in subtoxic concentrations, ADC did not influence cell viability in the absence of IFN-γ (Fig. 4B). Upon IFN-γ stimulation, which resulted in a NO-mediated decrease in cell survival, ADC inhibited NO release and partially restored the viability of fibrosarcoma cells (Fig. 4B). In contrast to ADC, paclitaxel significantly reduced the oxidative metabolism of unstimulated L929 cells (Fig. 4C). In IFN-γ-stimulated L929 cultures, however, paclitaxel diminished NO production and restored cell respiration to the levels

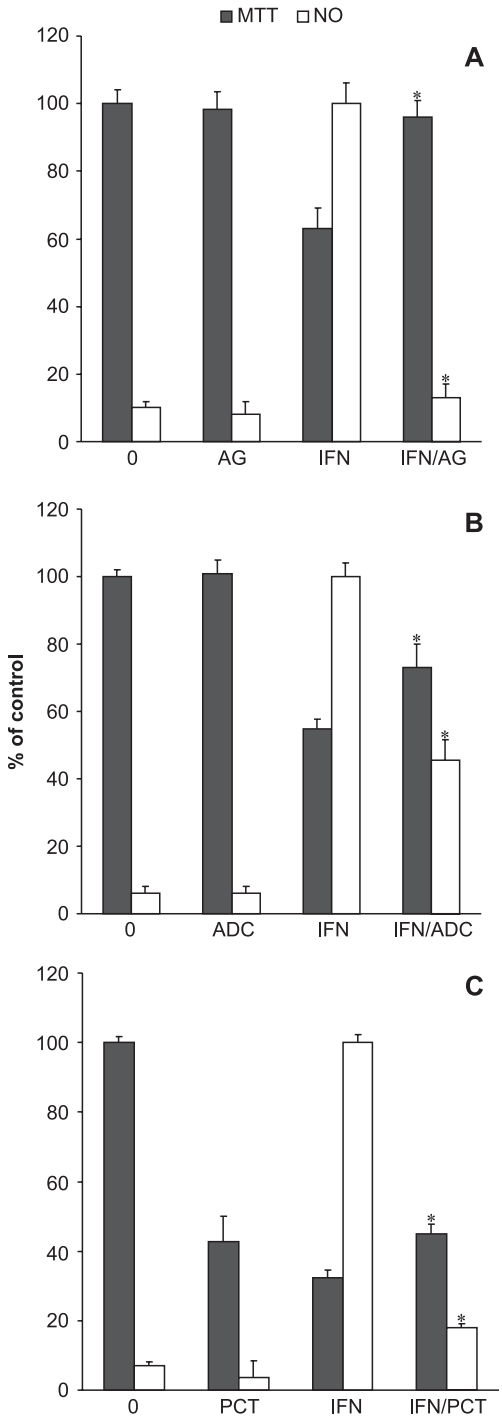

Fig. 4. ADC and paclitaxel interfere with NO-dependent autotoxicity in L929 cells. Unstimulated or IFN-γ-activated L929 cells were treated with 2 mM aminoguanidine (A), 5 μg/ml ADC (B), or 2.5 μM paclitaxel (PCT) (C) as indicated. Nitrite accumulation in cell culture supernatants and cell viability (MTT assay) were measured after 48 h of cultivation. Nitrite accumulation is presented as % of control values obtained from IFN-γ-stimulated cell cultures (23.32 ± 1.40, 27.71 ± 1.73, 20.59 ± 0.44 μM, for (A), (B), and (C), respectively), and cell viability is presented as % of control absorbance determined in cell cultures incubated in medium alone (0.893 ± 0.036, 0.699 ± 0.011, 1.050 ± 0.017, for (A), (B), and (C), respectively); *$P < 0.05$ refers to corresponding cultures without aminoguanidine, ADC or paclitaxel.

observed in the absence of IFN-γ (Fig. 4C). It therefore appears that L929 cells could benefit from ADC or paclitaxel-mediated inhibition of IFN-γ-induced NO production.

4. Discussion

The results presented here clearly demonstrate that the anti-cancer drugs ADC and paclitaxel are potent inhibitors of IFN-γ-triggered iNOS induction and subsequent production of NO in L929 fibrosarcoma cells. The observed reduction of endogenous NO release from fibrosarcoma L929 cells correlated with the increase in their survival, thus possibly having important implications for the therapeutic effects of the drugs investigated.

The inhibitory action of ADC and paclitaxel on NO release by L929 fibrosarcoma cells was not due to the transformed nature of the cells investigated, since it was also observed in primary fibroblasts and endothelial cells. Still, the inhibitory effect of the drugs was more profound in fibrosarcoma cells, thus suggesting that transformed cells might be more sensitive to the action of the drugs than their primary counterparts. In contrast, both drugs failed to prevent NO release by macrophages, thus indirectly confirming distinct requirements for iNOS activation in different cell types (Jankovic et al., 2000; Miljkovic et al., 2002, 2003; Rao, 2000). Neither paclitaxel nor ADC was able to suppress NO production in L929 cells when transcription was blocked, implying that iNOS gene expression was a likely target for the action of the drugs. Their ability to markedly reduce the amount of iNOS protein in IFN-γ-stimulated L929 cells indeed supports such an assumption. However, we could not completely exclude the possibility that the drugs might additionally affect the expression of iNOS at a post-transcriptional level or directly alter its enzymatic activity through some protein synthesis-dependent mechanism(s). While at least three signaling pathways, including activation of signal transducers and activators of transcription (STAT), nuclear factor-kappa B (NF-κB) and MAPK, control the IFN-γ-mediated induction of iNOS (MacMicking et al., 1997), the activation of p44/42 MAPK has been reportedly involved in the iNOS induction in fibroblasts (Wang and Brecher, 1999; Jankovic et al., 2000; Miljkovic et al., 2002, 2003). Our finding that ADC and paclitaxel effectively prevented IFN-γ-induced phosphorylation of p44/42 MAPK in L929 cells indicates that an impairment of p44/42 MAPK signaling might be partly responsible for the action of both agents. Such an assumption is consistent with our earlier observation that the specific p44/42 MAPK inhibitor 2-[2-Amino-3-methoxyphenyl]-4*H*-1-benzopyran-4-one (PD 098059) efficiently inhibited IFN-γ-triggered NO production in L929 cells, thus mimicking ADC and paclitaxel effects (Jankovic et al., 2000).

Since ADC is incorporated into newly synthesized DNA (Christman, 2002), it is unlikely that the DNA-demethylating action of the drug contributes to its interference with p44/42 MAPK activation, which was observed after only 30 min following IFN-γ stimulation. Still, as another DNA demethylating agent with a different mode of action, L-ethionine, also downregulated NO production in L929 cells, it is possible that ADC might impair NO synthesis through both DNA demethylation-dependent and demethylation-independent mechanisms. Demethylation could presumably influence iNOS gene expression directly, through modification of iNOS gene regulatory elements, as well as indirectly, through modulation of the expression of various molecules that control iNOS gene transcription. The latter assumption is consistent with the observation that more than 60% of ADC-affected genes in a colon cancer cell line did not have demethylation-dependent regulatory elements (Liang et al., 2002). However, one should be cautious when interpreting the data obtained with ethionine, since it is possible that it might affect NO production in L929 cells through demethylation-independent mechanism(s). As for the mechanisms behind paclitaxel-mediated suppression of p44/42 MAPK activation and iNOS expression in L929 fibroblasts, it is conceivable that the observed effects were, at least in part, due to the microtubule-stabilizing properties of the drug. This hypothesis is supported by data suggesting that microtubule association–dissociation dynamics might play an important role in the activity of MAPK (often called microtubule-associated protein kinases) (Schmid-Alliana et al., 1998), as well as in the optimal induction of iNOS (Kirikae et al., 1996; Marczin et al., 1996). Accordingly, in the present study, the microtubule-disrupting agent colchicine readily mimicked the inhibitory effect of paclitaxel on NO production in L929 cells, and has also been shown capable of inhibiting p44/42 MAPK activation (Shirakata et al., 1999; Stone and Chambers, 2000, and our unpublished observation). In contrast, paclitaxel is well known for its lipopolysaccharide-mimetic activity in macrophages, enhancing both MAPK activation and subsequent expression of iNOS (Ding et al., 1993; Byrd-Leifer et al., 2001; Manthey et al., 1994). The latter effect was confirmed in our study, indicating that stabilization of microtubules by paclitaxel might oppositely influence MAPK-controlled iNOS induction in different cell types.

There is a question of the possible biological significance of the findings presented here. As NO produced by macrophages has mostly been associated with cytotoxic actions against tumor cells (MacMicking et al., 1997), the inability of ADC and paclitaxel to block macrophage NO release presumably would not interfere with macrophage tumoricidal activity. Moreover, the paclitaxel-mediated enhancement of macrophage NO synthesis, confirmed in the present study, has been proposed to contribute to the anti-cancer action of the drug. However, our data suggest that interference of ADC and paclitaxel with iNOS induction in transformed cells might also have an adverse effect on the anti-tumor immune response, protecting tumor cells from NO-mediated suicide triggered by proinflammatory cytokines. However, the role of

tumor-derived, as well as endothelium-derived, NO in tumor progression is apparently much more complex, as it could also involve the promotion of tumor growth and metastasis through stimulation of angiogenesis and vasodilatation (Fukumura and Jain, 1998; Lala and Orucevic, 1998; Bogdan, 2001). Thus, the observed inhibition by ADC and paclitaxel of NO release from tumor cells and endothelium could also reduce tumor progression. The net effect of ADC and paclitaxel-mediated interference with iNOS-mediated NO production will therefore depend on several parameters, with the ability of tumor cells to synthesize NO, tumor cell sensitivity to NO, and the extent of macrophage infiltration arguably being the most important. An accurate prediction of the influence that iNOS modulation by ADC and paclitaxel may have on tumor cells is further complicated by the possibility that, regarding iNOS activation, different types of tumors might exhibit distinct responses to ADC or paclitaxel. Indeed, our preliminary results show that both agents, in contrast to their effect on fibroblasts and endothelial cells, markedly potentiated IFN-γ-mediated iNOS induction and subsequent NO release in primary astrocytes and astrocytoma cells (unpublished observation). Finally, since the excessive production of NO by fibroblasts and endothelial cells has been implicated in inflammatory joint diseases (McInnes et al., 1996) and septic shock (Holzmann, 1997), respectively, our results point to a possible anti-inflammatory action of ADC and paclitaxel. This is consistent with the results of some studies, which indicate the potential usefulness of ADC and paclitaxel in autoimmune disorders (Yoshida et al., 1990; Kroger et al., 1999; Brahn et al., 1994; Cao et al., 2000).

In conclusion, the present work suggests that the anticancer drugs ADC and paclitaxel, besides having a direct cytotoxic effect on tumor cells, might affect tumor cell biology indirectly, through interference with NO production. Since the in vitro reductionist approach alone is insufficient for unambiguous prediction of the effect that such an action might have on tumor progression, more complex in vivo studies and, possibly, mathematical modeling, are needed to accomplish this task.

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