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ORIGINAL ARTICLE

Gül Özcan Arican

Cytoprotective effects of amifostine and cysteamine on cultured normal and tumor cells treated with paclitaxel in terms of mitotic index and ^{3}H-thymidine labeling index

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Abstract *Purpose*: In this study, the cytotoxic effects of paclitaxel (PAC) in normal and tumor cells were established, and the cytoprotective effects of amifostine (AMI) and cysteamine (CYS) against this cytotoxicity were examined. *Methods*: Tumor cell lines used in this study were L-strain cells of mouse subcutaneous origin and human cervix carcinoma-derived HeLa cells. Mouse embryonic fibroblasts (MEFs) were used as the normal cell line. Results of the experiments were evaluated in terms of the mitotic index and the ^{3}H-thymidine labeling index. PAC concentrations of 6 and 12 μg/ml were applied to the cells for 1–10 days either alone or in combination with 1 μg/ml of AMI and CYS. *Results*: In terms of the above parameters, statistically significant effects were not seen in cultures of any of the cell lines treated with 1 μg/ml of AMI or CYS alone. In contrast, both concentrations of PAC caused increasing cytotoxic effects with increasing treatment time ($P < 0.001$). The cytotoxic effect of PAC appeared as mitotic phase accumulation (G_2/M blockage) and a subsequent decline in the synthesis phase. HeLa cells were very sensitive to PAC treatment, whereas MEF cells were quite resistant compared with tumor cells. In cells treated with combined drugs to investigate the cytoprotective effects of AMI and CYS on normal and tumor cell lines, PAC continued to show cytotoxic effects in tumor cells, but this effect was reduced in the normal cells. *Conclusions*: AMI and CYS did not protect tumor cells against the cytotoxic effects of PAC, but protection was observed in normal cells. Furthermore, the protection provided by AMI was stronger than that provided by CYS.

G. Özcan Arican
Department of Biology, Faculty of Science,
Istanbul University, Istanbul, Turkey
E-mail: gozcan@istanbul.edu.tr
Tel.: +90-212-4555700
Fax: +90-212-5280527

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Introduction

Paclitaxel (PAC) is an antimicrotubule chemotherapy agent and a diterpene isolated from the bark of the Western (Pacific) yew, *Taxus brevifolia*. PAC acts by promoting the assembly of microtubules from tubulin dimers and stabilizing microtubules by preventing depolymerization [20]. PAC also inhibits the transition from G_0 to S phase by disrupting tubulin in the cell membrane and/or by direct inhibition of the cytoskeleton interrupting intracellular transport and communications. This mechanism of action is unlike that of other cytotoxics [10].

Amifostine (AMI, WR-2721), a phosphorylated thiol, has demonstrated the unique ability to protect normal but not tumor tissue from cytotoxic damage induced by radiation therapy and chemotherapy. AMI selectively protects a broad range of normal tissues. This selective protection is based on differential dephosphorylation by alkaline phosphatase at tissue sites and the preferential uptake of the active thiol metabolite, WR-1065, by cells in normal tissue [24]. The cytoprotective effect of AMI is thought to be a consequence of its ability to scavenge reactive oxygen species and its apoptosis-inducing effect [4]. The apoptotic response of cells to an anticancer drug is mediated by wild-type p53. The role of p53 on the mechanism involved in the effect of AMI is controversial but appears to depend on the cell type. A recent study on myeloid leukemia cell lines showed that AMI impairs p53-dependent apoptosis by reducing the activation of apoptosis-related genes [1].

In addition to the cytoprotective effects of WR-1065, two additional metabolites, cysteamine (CYS, 2-mercaptoethylamine) and the symmetric disulfide WR-33278, have cytoprotective properties [2, 3, 9]. Most of the

experimental studies have been done on the radioprotective effects of CYS [19]. Since the radioprotective properties of CYS became known, some studies have been carried out on the effects of this compound on tumor cells [5, 25].

This study was conducted to determine whether the interaction of the cytoprotective agents AMI and CYS which have recently been introduced to the market with the chemotherapy agent PAC plays role in mitosis and DNA synthesis stages in both normal and tumor cells.

Materials and methods

Tumor cell lines

L-strain (CCL 1) cells and HeLa (CCL 2) cells were employed as tumor cell lines. L-strain cells used in this study were derived from mouse fibroblasts by in vitro malign transformation [22]. Cells were maintained in monolayer culture in medium-199 (M-199, Gibco Laboratories, N.Y.) containing 10% fetal bovine serum (FBS; Gibco), 100 μg/ml streptomycin and 100 IU/ml penicillin, and were passaged twice a week in an appropriate number of 25 cm^2 flasks, and the volume of the complete medium in each flask was made up to 12 ml. The doubling time of this cell line was 22.8 h [17]. HeLa cells were employed as other tumor cell line. The cell line was isolated in 1951 from a carcinoma of the cervix in a black female. The cells were grown in minimum essential medium (MEM; Gibco) containing 10% FBS, 100 μg/ml streptomycin and 100 IU/ml penicillin, and were passaged twice a week in an appropriate number of 25 cm^2 flasks, and the volume of the complete medium in each flask was made up to 12 ml. The doubling time of this cell line was 19 h [22].

Normal cell line

Mouse embryonic fibroblasts (MEF) cells were used as the normal cell line [7]. To produce MEF cultures, albino mice (BALB/c strain) were allowed to mate and the third day from the beginning of the experiment was considered as the beginning of the urge to mate. The appearance of the ventral plaque was accepted as day 0 of pregnancy. On the 13th day of pregnancy, the mice were killed by cervical dislocation, and the uteri were removed aseptically and placed in Hank's balanced salt solution (HBSS; Gibco). The inner organs and extremities of the embryos removed from the uteri were separated, and the bodies were placed in HBSS and washed three or four times until free of blood. The posterior skin of the embryos that contain embryonic fibroblasts was put in a beaker and cut extensively using sterile scissors until a viscous tissue slurry was obtained. The tissue was then trypsinized by adding 0.25% trypsin solution and keeping the beaker on a magnetic stirrer at 37°C for 20 min. Later, the tissue suspension was transferred to centrifuge tubes and spun for 3 min at 1500 rpm. The supernatant was removed, and a second trypsinization for another 20 min was carried out on the first fraction sedimented. The second fraction was used to inoculate sterile culture flasks (25 cm^2) containing M-199. After 24 h, the embryo culture was washed with HBSS and the medium was replaced with fresh medium. This procedure was repeated until a single cell layer was obtained in the primary culture. All the experiments were conducted on secondary cultures prepared after a single cell layer was formed [8]. The doubling time of this cell line was 30.6 h [27].

Cell Culture

Cells were removed from the surface of the culture flasks by the addition of 0.25% trypsin (Gibco) and centrifuged for 3 min at 1500 rpm. Following the addition of medium to the cell precipitate, cells were cultured in a 24-well tissue culture plate covered with 1.10^4 cells/ml and incubated in an atmosphere comprising 5% CO_2 and 95% air for 24 h. After 24 h incubation the medium was removed and replaced with medium containing the drugs at various concentrations.

Drug treatments

PAC (Taxol, Bristol-Myers Squibb), AMI (ethyol, U.S. Bioscience) and CYS (2-aminoethanethiol, C_2H_7NS, Sigma, Germany) were dissolved immediately before use in sterile medium to give the required concentration. The concentrations of PAC used were the IC_{30} and IC_{50} concentrations (PAC I and PAC II, 6 and 12 μg/ml, respectively), and AMI and CYS were used at 1 μg/ml. Tumor and normal cells were treated with these doses for 0, 1, 2, 3, 4, 6, 8 and 10 days and were exposed to AMI, CYS, PAC I and PAC II, and the combinations PAC I + AMI, PAC I + CYS, PAC II + AMI and PAC II + CYS.

Mitotic index analysis

Mitotic indices were determined by the Feulgen method. Before the cells were treated with Schiff's reagent, they were prepared with 1 *N* HCl at room temperature for 1 min and then hydrolyzed with 1 *N* HCl for 10.5 min at 60°C. The slides were treated with Schiff's reagent, rinsed for a few minutes in distilled water and stained with 10% Giemsa solution, pH 6.8, for 3 min, and washed twice in phosphate buffer. After staining, the slides were rinsed in distilled water and air-dried. Finally, the mitotic indices were calculated by counting metaphases, anaphases and telophases for each tested drug concentration and control. At least 3000 cells were examined from each slide for determination of the mitotic index by the same scorer.

^{3}H-thymidine labeling

For each cell line, nine groups of experiments were performed. The control group did not receive any drugs, but the other eight groups received drugs on day 0 (inoculation day was considered as day 1). Cells were incubated in the presence of the drugs for 1, 2, 3, 4, 6, 8 or 10 days, transferred to fresh drug-free medium supplemented with 1 μCi/ml ^{3}H-thymidine (TRA-120, Amersham, Germany, 1 mCi/ml), and incubated for 30 min for determination of the labeling index.

Autoradiography

After labeling, cells were fixed Carnoy's fixative (3:1 mixture of ethanol/glacial acetic acid) and the remaining radioactive material was washed twice with 2% perchloric acid at 4°C for 30 min. After preparing the slides, they were coated with EM-1 gel emulsion (Amersham) prepared with distilled water at 40°C to determine the thymidine labeling index. After 3 days exposure at 4°C autoradiograms were washed with D-19 b developer (Kodak, Japan) and fixed with Fixaj B (Kodak). The slides were evaluated after Giemsa staining for 3 min. The same person evaluated all the slides by counting at least 3000 cells from each slide. The labeling index was calculated by examining 100 areas on each slide at a magnification of ×12.5.

Statistical analysis

The data were analyzed statistically using one- and two-sample directional Student's *t*-tests ($n = 100$). The data analyzed were those from a minimum of three independent experiments. Significance was accepted for *P* values < 0.01.

Results

L-strain cells

Effects of AMI and CYS alone

The mitotic and labeling indices of L-strain cells treated with 1 μg/ml of AMI or CYS alone are shown in Figs. 1 and 2. There were no statistically significant effects, as seen in the figures.

Effects of PAC I treatment either alone or in the presence of AMI or CYS

Treatment with PAC I (6 μg/ml) alone for 1–10 days significantly increased the mitotic index in L-strain cells with increasing treatment time ($P < 0.001$). Combination treatments also significantly increased the mitotic index in L-strain cells ($P < 0.001$; Fig. 3). Treatment with PAC I either alone or in the presence of AMI or CYS significantly decreased the labeling index in L-strain cells ($P < 0.001$). As shown by the labeling index results (Fig. 4), AMI and CYS had some protective effect in L-strain cells treated for 0–4 days ($P < 0.01$), but had no protective effect in cells treated for 6–10 days.

Effects of PAC II treatment either alone or in the presence of AMI or CYS

Treatment with PAC II (12 μg/ml) increased the percentage of cells in M phase, as was also seen with PAC I treatment. These increases were statistically significant when compared to control ($P < 0.001$). In addition, the effects of PAC II treatment were greater than those of PAC I treatment ($P < 0.001$). When AMI or CYS was added to PAC II, mitosis increased with respect to the

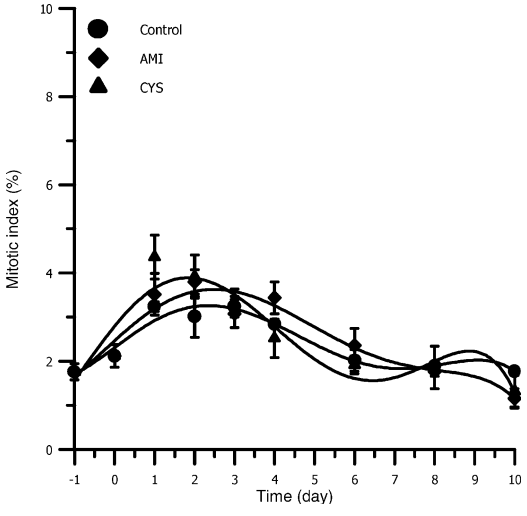

Fig. 1 The mitotic index in L-strain cells following treatment with 1 μg/ml of AMI and CYS alone

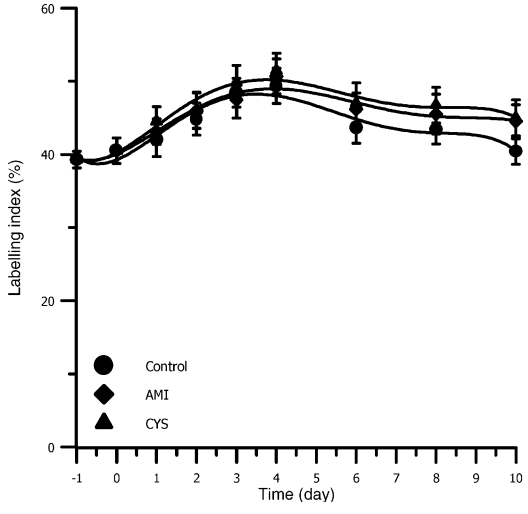

Fig. 2 The ^{3}H-thymidine labeling index in L-strain cells following treatment with 1 μg/ml of AMI and CYS alone

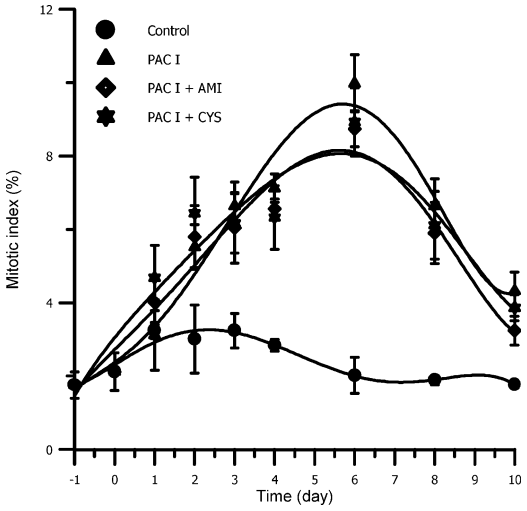

Fig. 3 Mitotic index values in L-strain cells treated with PAC I (6 μg/ml) either alone or in the presence of AMI or CYS

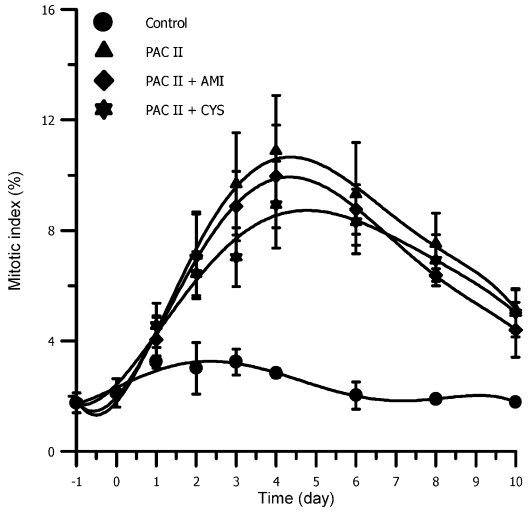

Fig. 5 Mitotic index values in L-strain cells treated with PAC II (12 μg/ml) either alone or in the presence of AMI or CYS

control cells ($P < 0.001$). There was no significant protective effect of the combination treatments when compared to the single treatments, as seen in Fig. 5. PAC II decreased the percentage of cells in S phase, as seen following PAC I treatment. This decrease was statistically significant when compared to control ($P < 0.001$). In addition, the inhibition of DNA synthesis was higher following PAC II treatment than following PAC I treatment ($P < 0.001$). When AMI or CYS were added to PAC II, DNA synthesis decreased with respect to the control cells ($P < 0.001$). At early time points (1–4 days), the labeling index was increased with respect to that following PAC II treatment alone. There was no significant protective effect of combined treatment for 6–10 days when compared to single treatment, as seen in Fig. 6.

HeLa cells

Effects of AMI and CYS alone

Treatment with AMI or CYS alone had no significant effect on the mitotic index or labeling index of HeLa cells compared to controls (Figs. 7 and 8).

Effects of PAC I treatment either alone or in the presence of AMI or CYS

The mitotic index in HeLa cells treated with PAC I (Fig. 9) revealed that PAC exerts its cytotoxic effect by mitotic cell accumulation. Comparing PAC I treatment alone and in combination with AMI or CYS, there were significant differences with respect to control ($P < 0.001$).

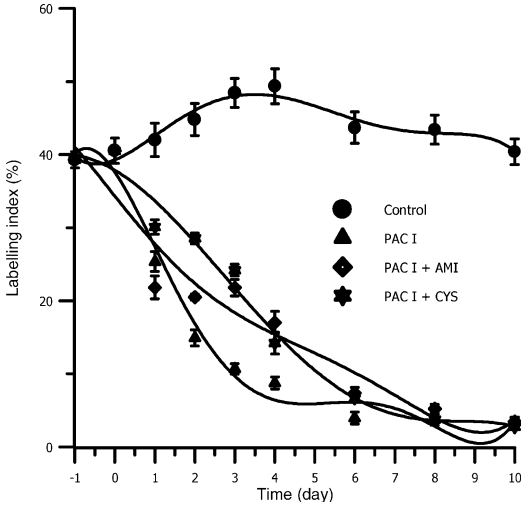

Fig. 4 ^{3}H-Thymidine labeling index in L-strain cells treated with PAC I (6 μg/ml) either alone or in the presence of AMI or CYS

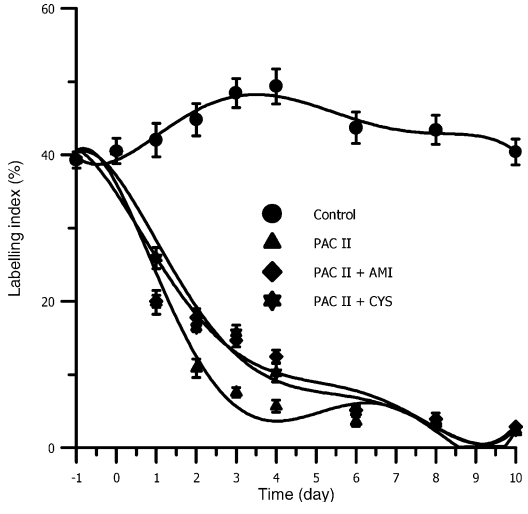

Fig. 6 ^{3}H-Thymidine labeling index in L-strain cells treated with PAC II (12 μg/ml) either alone or in the presence of AMI or CYS

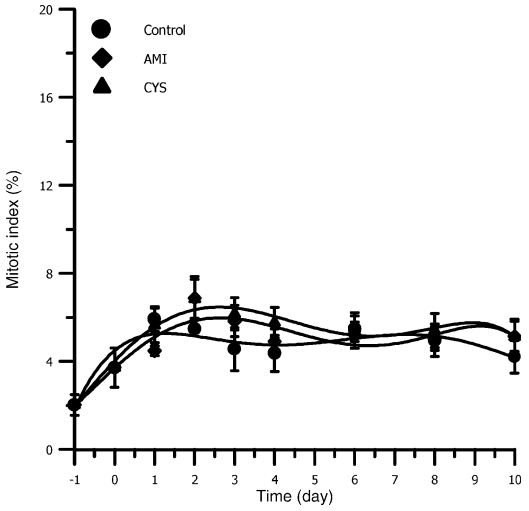

Fig. 7 Mitotic index in HeLa cells treated with 1 μg/ml of AMI or CYS

The labeling index in HeLa cells treated with PAC I (Fig. 10) revealed that PAC exerts its cytotoxic effect by inhibiting DNA synthesis. Comparing PAC I treatment alone and in combination with AMI or CYS, there were significant differences with respect to control ($P < 0.001$). AMI and CYS did not protect HeLa cells.

Effects of PAC II treatment either alone or in the presence of AMI or CYS

The effects of drug treatment on the mitotic index in HeLa cells are shown in Fig. 11. Drug treatment caused a significant accumulation of mitotic HeLa cells with respect to control ($P < 0.001$). Treatment with PAC II alone had a greater effect than treatment with PAC II in combination with AMI or CYS. The effects of treatment

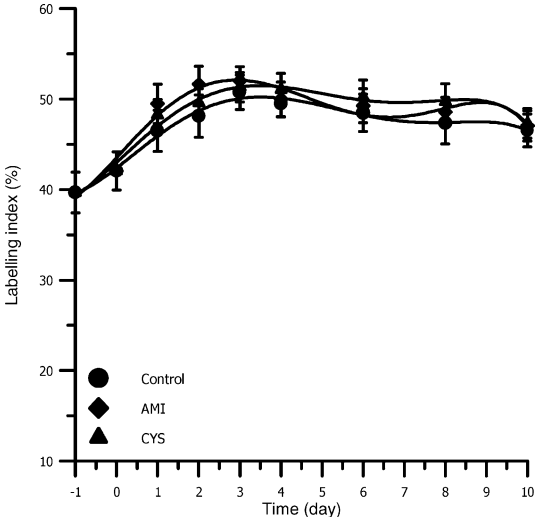

Fig. 8 ^{3}H-Thymidine labeling index in HeLa cells treated with 1 μg/ml of AMI or CYS

on the mitotic index in HeLa cells were greater than those in L-strain cells. There was also no significant protective effect in HeLa cells of AMI or CYS in combination with PAC II. The labeling index was significantly decreased with respect to control ($P < 0.001$; Fig. 12).

Mouse embryonic fibroblast cells

Effects of AMI and CYS alone

Treatment with AMI or CYS alone had no significant effect on the mitotic index or labeling index of MEF cells compared to controls (Figs. 13 and 14).

Effects of PAC I treatment either alone or in the presence of AMI or CYS

The mitotic index in normal MEF cells treated with PAC I alone significantly increased with increasing treatment times with respect to controls ($P < 0.001$), as shown in Fig. 15. In combination with AMI or CYS, the cytotoxic effects of PAC I treatment were reduced. Comparing treatment with PAC I alone and in combination with AMI or CYS, significant differences were found ($P < 0.001$). The protective effect of AMI was higher than that of CYS ($P < 0.001$). The labeling index in normal MEF cells treated with PAC I alone significantly decreased with increasing treatment times with respect to controls ($P < 0.001$), as seen in Fig. 16. In combination with AMI or CYS, the toxic effect of PAC I treatment was significantly reduced ($P < 0.001$).

Effects of PAC II treatment either alone or in the presence of AMI or CYS

The effects of the drug treatments on the mitotic index in MEF cells were shown in Fig. 17. PAC II treatment increased the mitotic index in MEF cells with increasing treatment time compared to controls ($P < 0.001$). The effect of PAC II in combination with either AMI or CYS was significantly different ($p < 0.001$) from that of PAC II treatment alone. CYS added to PAC II provided a significant protective effect in normal MEF cells compared to controls ($p < 0.001$). There was no significant difference in mitotic index in MEF cells between control cells and those treated with PAC II in combination with AMI. Thus, the protection by AMI was stronger than that by CYS ($P < 0.001$), and AMI selectively protected the normal MEF cells. PAC II treatment significantly decreased ($P < 0.001$) the labeling index in MEF cells with increasing treatment time compared to controls, as being shown in Fig. 18. The addition of either AMI or CYS to PAC II reduced PAC II's toxicity. Either of these combinations protected the normal MEF cells as seen in terms of the mitotic index.

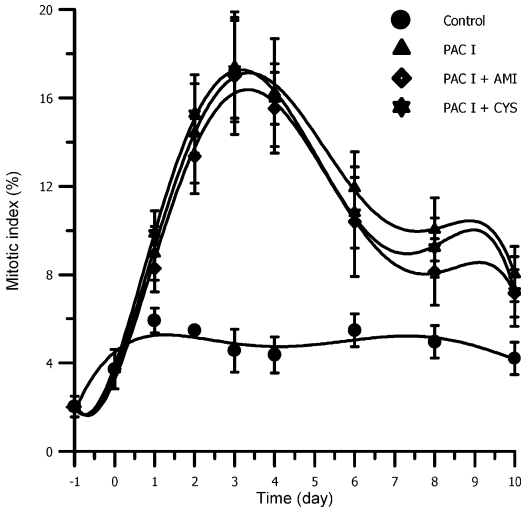

Fig. 9 Mitotic index in HeLa cells treated with PAC I (6 μg/ml) either alone or in the presence of AMI or CYS

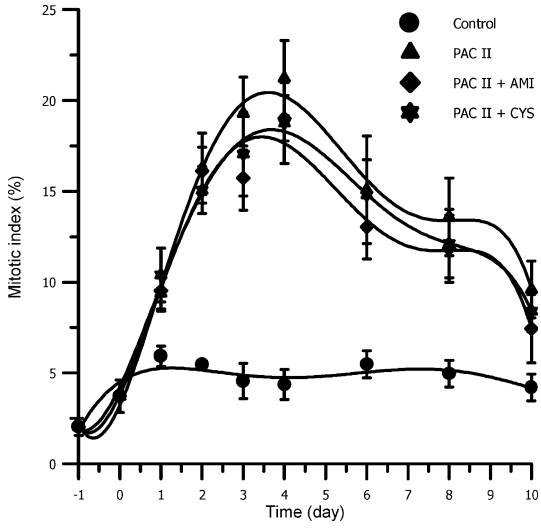

Fig. 11 Mitotic index in HeLa cells treated with PAC II (12 μg/ml) either alone or in the presence of AMI or CYS

Discussion

In this study, the cytotoxic effects of PAC in normal and tumor cells were investigated, and the cytoprotective effects of AMI and CYS against this cytotoxicity were examined.

Tumor and normal cells treated with AMI or CYS showed no significant differences in mitotic index and labeling index compared to untreated cells (Figs. 1, 2, 7, 8, 13, 14). In all three cell lines, PAC I and PAC II treatments exerted their cytotoxic effects by causing accumulation of mitotic cells and a decrease in the proportion of labeled cells with increasing time. The accumulation of cells resulted in an increase in the mitotic index, which was an expected consequence of PAC treatment. The mitotic index increased in all cell lines due to PAC treatment but then appeared to decrease after reaching a peak value. This drop could suggest that the cells, which accumulated in mitosis phase were still under the influence of the drug, because even the minimum mitotic index was significantly higher than that of controls ($P < 0.001$). This drop could be interpreted as an indication of cell death caused by PAC.

In all the cell lines used, the decreases in labeling index were proportional to the increases in mitotic index values caused by PAC. Therefore, the decrease in labeling index values was a result of accumulation of cells in mitosis rather than the effect of the drug on the synthesis phase. As a result, cells could not proceed to the synthesis phase and the labeling index values decreased as expected. Thus, the cytoprotective effects of AMI and CYS in these cells are valid in terms of the labeling index criterion as well.

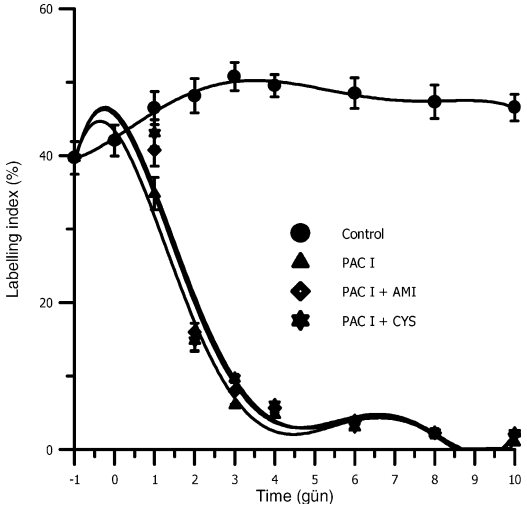

Fig. 10 ^{3}H-Thymidine labeling index in HeLa cells treated with PAC I (6 μg/ml) either alone or in the presence of AMI or CYS

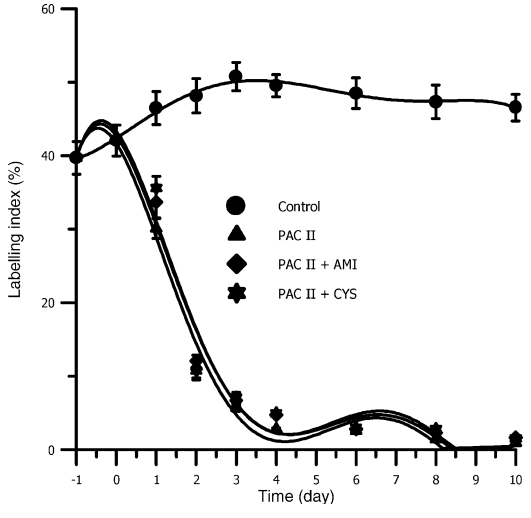

Fig. 12 ^{3}H-Thymidine labeling index in HeLa cells treated with PAC II (12 μg/ml) either alone or in the presence of AMI or CYS

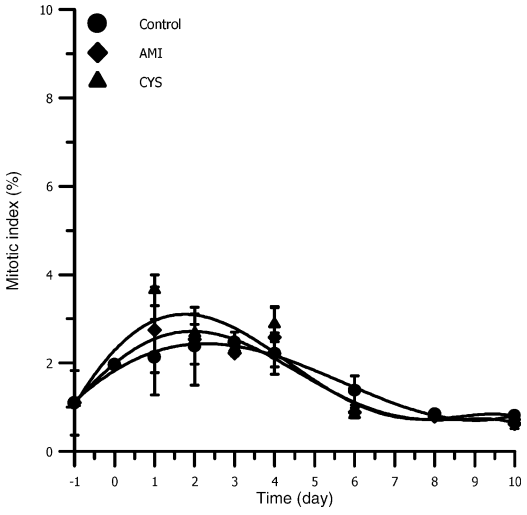

Fig. 13 Mitotic index in MEF cells treated with 1 μg/ml of AMI or CYS

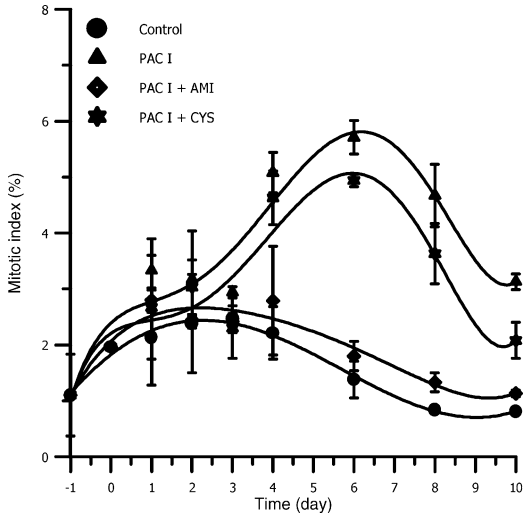

Fig. 15 Mitotic index in MEF cells treated with PAC I (6 μg/ml) either alone or in the presence of AMI or CYS

In the tumor cell lines, the maximum cytotoxic effect of PAC was found in HeLa cells. Besides, PAC in combination with AMI or CYS produced different effects according to the cell line. In normal MEF cells, the toxic effect of PAC was reduced when PAC was used in combination with AMI or CYS. Although AMI was shown to be effective in protecting normal cells, in tumor cell lines (L-strain or HeLa cells) no such effect was found.

The cytoprotective effects of AMI or CYS and the toxicity exerted by PAC alone appeared in the same time period. The protection provided by AMI was again stronger than that by CYS. The cell lines used in the current study showed different levels of sensitivity as well. In comparison to tumor cells, MEF cells appeared more resistant to the increase in mitotic phase accumulation and the decrease in the proportion of labeled cells caused by PAC (Figs. 15, 16, 17 and 18). During treatment days 0–4 in L-strain cells, AMI and CYS had significant protective effects ($P < 0.01$), but during the later treatment time (days 6–10), this protective effect disappeared (Figs. 4 and 6). HeLa cells were the most sensitive of the tumor cells at early time points (Figs. 10 and 12). The differences in sensitivity could be explained by the differences in the cell type and cell cycle length, and the cells may also have been affected differently because of differences in the phase distribution of the cells. Indeed, it has been shown that cells with a shorter cell cycle are more sensitive to antineoplastic drugs. HeLa cells divide two times faster than L-strain cells, and hence are more sensitive [11, 16].

Laboratory studies have shown that AMI possesses broad-spectrum selective cytoprotective properties and

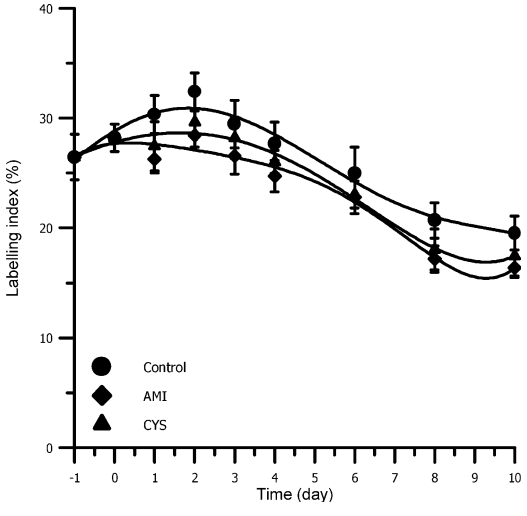

Fig. 14 ^{3}H-Thymidine labeling index in MEF cells treated with 1 μg/ml of AMI or CYS

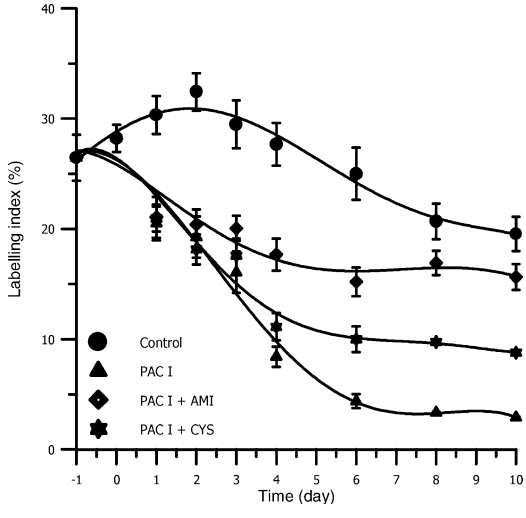

Fig. 16 ^{3}H-Thymidine labeling index in MEF cells treated with PAC I (6 μg/ml) either alone or in the presence of AMI or CYS

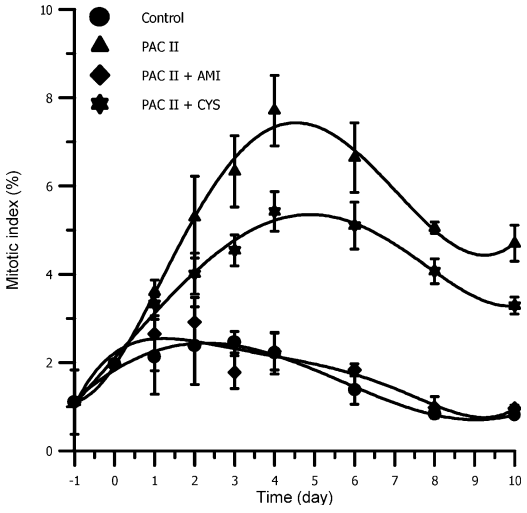

Fig. 17 Mitotic index in MEF cells treated with PAC II (12 μg/ml) either alone or in the presence of AMI or CYS

can protect against the toxicity of other classes of anticancer agents, such as anthracyclines and taxanes [15, 21]. This protective effect occurs in normal tissues and has been found to be due to the presence of high phosphatase activity, neutral pH, and more efficient vessel formation in normal tissues in contrast to tumor tissues. AMI is used as a premedication. It is phosphorylated by alkaline phosphatase, converted to its active metabolite, free thiol, and reduces the toxicity of chemotherapeutic drugs. Under the conditions mentioned above, free thiol attaches to and detoxifies the reactive metabolite of the drug, and it can also remove the free radicals formed in the tissues receiving this drug [24]. Although the cellular target of PAC is tubulin, not DNA, preclinical studies have shown that AMI may offer selective cytoprotection of normal tissues from PAC as well [13]. In addition, AMI reduces the number of DNA single-strand breaks

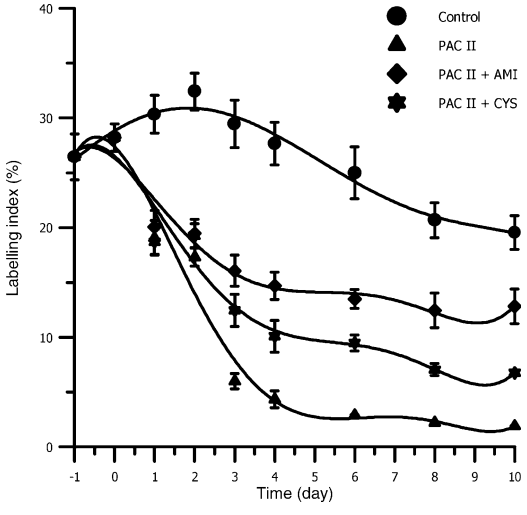

Fig. 18 ^{3}H-Thymidine labeling index in MEF cells treated with PAC II (12 μg/ml) either alone or in the presence of AMI or CYS

in MRC cells induced by PAC but does not decrease the number of single-strand breaks in A427 cells. In an in vivo system, AMI plus PAC has been shown to have a greater antitumor effect than PAC alone when tested in a murine ovarian cancer model [18]. Additionally, WR-1065 protects normal human diploid fibroblasts from PAC toxicity, while fibrosarcoma cells are unaffected or sensitized, suggesting a less straightforward explanation for the therapeutic effect of this drug [26, 28].

In transformed cells, WR-1065 protects cells from the cytotoxic effects of PAC in a p53-dependent manner. However, in a transformed human tumor cell line, WR-1065 provides no protection, consistent with the premise that p53-dependent growth arrest is the basis for the protective effect of this compound, and that this pathway is abrogated in human tumors [23]. The molecular basis of this effect is not clear, but a recent study has indicated that AMI-induced G_1 arrest and cytoprotection are mediated via a pathway that is dependent on p53 protein and that AMI-induced expression of p21 protein is not sufficient to sustain a G_1 arrest or to mediate cytoprotection. However, it is not known whether AMI directly affects p53-induced cell death or whether p53 protein is involved in resistance to AMI-induced apoptosis [12]. It is generally accepted that changes in gene expression in target cells can lead to an acquired resistant phenotype and can also provide clues about intracellular drug targets [4]. The reason for AMI's selectivity in its protective effect in MEF cells in terms of mitotic and labeling index criteria mentioned above can be explained by these mechanisms.

In vitro and in vivo laboratory studies have shown that CYS can increase the mitotic activity of rat, mouse and human tumor cell lines. These studies suggest that a biological protection mechanism might be involved in the mitotic activity of normal or malignant tissue cells [5, 6, 14, 24]. In the experimental model used in this study, CYS was shown to possess a significant cytoprotective activity. However, when the combinations of PAC + AMI and PAC + CYS were compared, AMI was found to provide more effective protection ($P < 0.001$). The cell kinetic parameters in this study provided limited information to explain the cellular mechanisms of the cytoprotective effect. Besides, the results concerning the cytoprotective effect demonstrated that more detailed parameters must be investigated in order to evaluate the level of these effects. Nevertheless, the time-dependent increases in the level of cytotoxicity and mitotic phase accumulation, and decreases in labeling index, following PAC treatment are in good agreement with the results of previous studies [2, 9, 13, 19, 26]. Particularly, the demonstration of the protective activity of AMI, which was selectively exerted in normal cells is the most important finding of this study.

The results obtained in this study could play an important role as a resource when designing other in vivo and in vitro experiments with various cell groups, which altogether may lead to better tumor therapy programs.

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