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Antifolates can potentiate topoisomerase II inhibitors in vitro and in vivo

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Abstract Antifolates have been shown to increase the DNA strand breaks produced by the topoisomerase inhibitor etoposide. PT523 is a potent new antifolate that cannot be polyglutamated. Human SCC-25 squamous carcinoma cells were exposed to methotrexate, trimetrexate or PT523 at a concentration of 5 μ*M* for 24 h along with various concentrations of etoposide or novobiocin during the final 2 h. Isobologram analysis of the treatment combinations indicated that exposure of the cells to PT523/etoposide, methotrexate/etoposide, PT523/novobiocin, methotrexate/novobiocin and trimetrexate/novobiocin resulted in greater than additive cytotoxicity. DNA alkaline elution studies with the same drug combinations indicated that there were three- to four-fold increases in the radiation equivalent (rad equivalent) strand breaks in the cellular DNA with etoposide or novobiocin along with the antifolate compared with the topoisomerase II inhibitors alone. Tumor growth delay studies were carried out in the murine SCC VII squamous carcinoma. PT523 (0.5 mg/kg) and methotrexate (2 mg/kg) were administered by 7-day continuous infusion while trimetrexate (3.75 mg/kg) was administered intraperitoneally daily on days 7–9. Etoposide (10 mg/kg) and novobiocin (100 mg/kg) were administered intraperitoneally on alternate days (7, 9, 11). The combinations of PT523 with etoposide or novobiocin were significantly more effective than methotrexate and etoposide or novobiocin, producing tumor growth delays of 8.4 days and 6.9 days, respectively. Overall, the antifolate/topoisomerase II inhibitor treatment combinations produced tumor growth delays that were apparently additive to greater than additive.

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Introduction

The antifolates, aminopterin and methotrexate, were among the earliest very successful anticancer drugs to enter clinical use [28]. The biochemical pathways inhibited and/or altered by exposure of cells to methotrexate have been elucidated over the past 30 years [9, 44, 45]. The major enzyme target of the antifolates is dihydrofolate reductase. Inhibition of dihydrofolate reductase leads indirectly to inhibition of thymidylate synthase, thus depleting cellular thymidylate. Inhibition of dihydrofolate reductase also leads to depletion of reduced folates in cells, which indirectly results in decreased purine biosynthesis. Like folic acid, the natural substrate for dihydrofolate reductase, methotrexate, is retained in cells by polyglutamylation of the monoglutamate side-chain of the molecule. Extensive studies have been carried out to characterize the biochemical basis of both intrinsic and acquired methotrexate resistance. Mechanisms of resistance identified thus far include diminished drug uptake, defective polyglutamylation, increased production of dihydrofolate reductase, production of a mutant dihydrofolate reductase with low methotrexate affinity, and increased salvage of DNA precursors [5, 52].

Newer antifolates have been designed in part to overcome or bypass the mechanisms by which cells become resistant to methotrexate. Trimetrexate is a "non-classical" antifolate that differs from methotrexate in its transport and intracellular retention, and may be useful against tumors resistant to methotrexate because of impaired transport or deficient polyglutamylation [32]. PT523 is a new antifolate which cannot be polyglutamylated. PT523 has potent activity against tumor cell lines in culture, including cells that

are 20- to 30-fold resistant to methotrexate by virtue of either a defect in transport or an increase in dihydrofolate reductase content [42, 46–48].

The antifolates interact with several biochemical pathways that are critical in cells. The ultimate lethality caused by exposure to antifolates appears to be activation of the pathway of apoptosis so that exposure to antifolates ultimately induces DNA strand breaks [2, 15, 16, 25, 27, 29, 31, 35, 41, 51].

Chromosomal DNA is maintained in a supercoiled state via attachment to the nuclear matrix [40, 60]. During active processes such as DNA replication, transcription, recombination, and repair, the DNA topoisomerases I and II are required for unwinding of the supercoiled structure [12, 37, 60, 61, 64]. Topoisomerase II cleaves both strands of DNA, becomes covalently bound to a 5′-phosphoryl end of the DNA [61] and allows the passage of a double helix of DNA before resealing the strand breaks [23, 34, 62], a process that requires adenosine 5′-triphosphate (ATP) hydrolysis. Experimental evidence thus far supports a direct role for topoisomerase II in DNA organization and replication [1, 4, 14, 19, 22, 26, 39, 66]. The anticancer drug etoposide, a semisynthetic derivative of podophyllotoxin, binds to topoisomerase II and DNA form to a ternary complex [50, 61, 62, 68]. Novobiocin is also an inhibitor of topoisomerase II, binding to the enzymes at the ATP binding site. Novobiocin does not produce a cleavable complex, that is topoisomerase II concealed DNA strand breaks, as does etoposide. It has myriad effects that occur at pharmacologically relevant concentrations, and cell killing by novobiocin is likely to involve multiple mechanisms [8, 13, 17, 24, 30, 38, 59].

As for the antifolates, it appears that ultimately, the lethality of topoisomerase II inhibitors, especially etoposide, in cells is believed to be due to activation of the pathway of apoptosis resulting in DNA strand breaking [2, 16, 27, 29, 51].

Several reports have appeared examining combinations of antifolates and topoisomerase II inhibitors in cell culture [18, 21, 36]. The current study was undertaken to compare PT523 [46–49] with methotrexate and trimetrexate in combination with topoisomerase II inhibitors in cell culture and in vivo.

Materials and methods

Drugs

Methotrexate, as the disodium salt, was a generous gift from Lederle Laboratories (Pearl River, N.Y.). 2,4-Diamino-5-methyl-6-[(3, 4, 5-trimethoxyanilino)methylamino]-quinazoline (trimetrexate), as the glucuronate salt, was kindly provided by Drs. D. Fry and R. Jackson (Warner Lambert, Ann Arbor, Mich.). *N*α-(4-Amino-4-deoxypteroyl)-*N*δ-hemiphthaloyl-L-ornithine (PT523) was prepared in our laboratory as described previously [46]. Etoposide and novobiocin were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Cell line

The human SCC-25 squamous carcinoma of the head and neck cell line is epitheloid in appearance and grows without the aid of a feeder layer [43]. It has a plating efficiency of 10–35%. The cells grow in DMEM supplemented with 10% fetal bovine serum, antibiotics and hydrocortisone (0.4 μg/ml) [20]. The generation time of SCC-25 cells is 48 h [58].

Cell survival studies

SCC-25 cells in exponential growth were exposed to various concentrations (0.01, 0.1, 0.5, 1, 5 or 10 μ*M*) of PT523, methotrexate or trimetrexate for 24, 48, 72 or 96 h in medium with serum. In another series of experiments, SCC-25 cells in exponential growth were exposed to various concentrations of etoposide (1, 5, 10, 50 or 100 μ*M*) or novobiocin (10, 50, 100, 250 or 500 μ*M*) for 2 h alone, or for the last 2 h of a 24-h treatment with 5 μ*M* PT523, methotrexate or trimetrexate.

Following the drug treatments, the cells were washed with phosphate-buffered 0.9% saline, detached with 0.25% trypsin and plated in duplicate at three dilutions for colony formation. After 2 weeks the colonies were visualized by staining with crystal violet, and colonies of 50 cells or more were counted. Results were expressed as the surviving fraction of treated cells compared with vehicle-treated control cells [55, 56, 58].

DNA alkaline elution

Alkaline elution was performed by standard procedures [58, 69, 70]. Samples were counted by liquid scintillation on an LS 7000 Beckman scintillation counter. Each point was measured in three independent experiments. DNA strand breaks were expressed as rad equivalents. Rad equivalents represent the radiation dose (rad) required to produce the same level of DNA strand breaking as determined by DNA alkaline elution after the chemotherapy treatments. SCC-25 cells treated with various doses of radiation (100, 200, 300, 400, 500 or 600 rad) were included in the same experiment as the drug treatments.

Tumor

The SCC VII squamous carcinoma is carried in male C3H mice (Jackson Laboratory, Bar Harbor, Me.). SCC VII is an in vivo/in vitro tumor line, and therefore can be used for the tumor cell assay. These cells grow in αMEM supplemented with 10% fetal bovine serum and antibiotics [6, 7, 67]. For the experiments, 2×10^6 tumor cells prepared from a brei of several stock tumors were implanted subcutaneously into a hind leg of male C3H mice 8–10 weeks of age.

Tumor growth delay experiments

Treatment was initiated on day 7 after tumor cell implantation, when the tumor volume was about 100 mm^3. PT523 (0.5 mg/kg) and methotrexate (2 mg/kg) were administered by continuous infusion over 7 days via subcutaneously implanted Alzet pumps (Alza Corp., Palo Alto, Calif.). Trimetrexate (75 mg/kg) was administered by intraperitoneal injection on days 7, 8 and 9. Etoposide (10 mg/ kg) and novobiocin (100 mg/kg) were administered by intraperitoneal injection on days 7, 9 and 11. The progress of each tumor was measured three times weekly until it reached a volume of 500 mm^3. Tumor growth delay was calculated as the number of days (± SE) required for each tumor to reach this volume as compared with

untreated controls. Each treatment group comprised five animals and each experiment was repeated three times.

Data analysis

Quantitative analysis of survival curves was carried out using the log-probit iterative least-squares method of Litchfield and Wilcoxon [33] as revised by Tallarida and Murray [54]. The method of Deen and Williams [10] was used to generate isobolograms for the special case in which the dose of one agent is held constant. This method produces envelopes of additive effect for different levels of the variable agents. It is conceptually identical to generating a series of isobologram and replotting the results at a constant dose of one agent on a log effect by dose of the second agent in a coordinate system. Dose–response curves were first generated for each individual agent using dose or log dose and effect, log effect, probit percentage of effect or logit percentage of effect relations. For cell survival dose response curves, correlations of 0.96 were obtained. The envelopes of additivity were generated from a series of isoeffect curves derived from the complete dose–response curves for each agent. Combinations producing the desired effect that lie within the envelope boundaries are considered to be additive; those displaced to the left are supra-additive and those displaced to the right are subadditive [3, 11, 53, 57].

Results

The survival of SCC-25 human squamous carcinoma cells exposed to each of the antifolates for various periods of time from 24 h to 96 h is shown in Fig. 1. The generation time of SCC-25 cells is 48 h [58]. Over the 3-log concentration range examined all three of the antifolates were broadly cytotoxic. PT523 and trimetrexate were more cytotoxic than methotrexate at each time point at the lowest concentration (0.01 μM) examined. Although each of the antifolates became more cytotoxic as the duration of drug exposure increased, this effect was most prominent with methotrexate. The potency of PT523 was manifest at the concentration of each antifolate required to kill 50% of the cells (Table 1). As the exposure time to PT523 was increased from 24 h to 96 h, there was a 600-fold decrease in the concentration of the drug required to kill 50% of the cells, while with methotrexate and trimetrexate this decrease was only 10-fold and 250-fold, respectively.

Etoposide was effectively cytotoxic towards SCC-25 cells, killing about 2-logs of cells upon exposure to 100 μM for 2h (Fig. 2). For combination studies the SCC-25 cells were exposed to 5 μM of each antifolate for 24 h with the addition of various concentrations of the topoisomerase II inhibitor during the last 2 h. The surviving fractions obtained for combinations of PT523 and etoposide showed greater than additive SCC-25 cell killing over the concentration range from 1 to 50 μM etoposide and additive cell killing with 100 μM etoposide. Combinations of methotrexate and etoposide resulted in cell killing that was additive at all doses of etoposide from 1 to 100 μM. Trimetrexate and etoposide in combination produced greater than additive SCC-25 cell killing over the etoposide concentration range from 1 to 50 μM and additive cell killing with 100 μM etoposide.

Novobiocin was much less cytotoxic than etoposide and a 2-h exposure to 500 μM novobiocin killed about 50% of the SCC-25 cells (Fig. 3). The combination of PT523 (5 μM) and 10–500 μM novobiocin resulted in greater than additive SCC-25 cell killing. While the combination of 5 μM methotrexate and 1–100 μM etoposide was additive in cytotoxicity, the combination of 5 μM methotrexate and novobiocin was markedly synergistic in cytotoxicity. There was more than 1 log more cell killing with the combination of methotrexate and novobiocin than would be expected if cell killing by the drugs were additive. Trimetrexate and novobiocin in combination produced greater than additive SCC-25 cell killing over the novobiocin concentration range from 10 to 250 μM novobiocin and additive cytotoxicity at the highest novobiocin concentration (500 μM) tested.

Exposure of cells to either antifolates or topoisomerase II inhibitors produces strand breaks in DNA. DNA alkaline elution was used to determine whether increased DNA strand breaks could be detected in cells exposed to the antifolate/topoisomerase II inhibitor combinations compared with the topoisomerase II inhibitors alone (Table 2). DNA strand breaks were expressed as radiation equivalents (rad equivalents) from radiation dose/elution curves. There was a general correlation between SCC-25 cell killing and rad equivalents of DNA strand breaks for the drugs and drug combinations except for the combination of methotrexate and novobiocin. Etoposide (50 μM, 2 h) killed about 1 log of SCC-25 cells and produced DNA strand breaks equivalent to 155 rad. The combinations of each

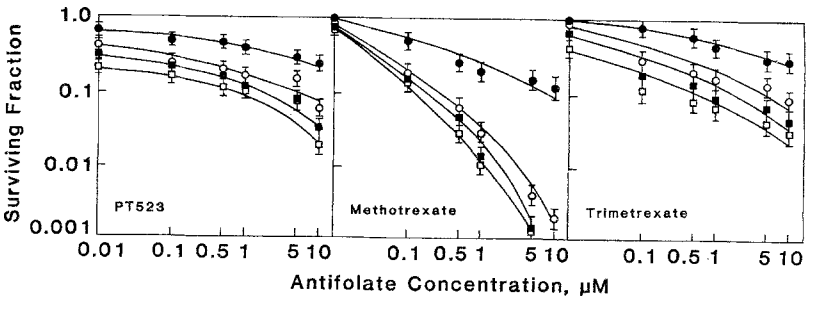

Fig. 1 Survival of human SCC-25 cell (derived from squamous carcinoma of the head and neck) exposed for 24 h (●), 48 h (○), 72 h (■) or 96 h (□) to the antifolates, PT523, methotrexate or trimetrexate. The generation time for SCC-25 cells was about 48 h. The values are the means of three experiments ± SEM

Table 1 Concentrations of antifolates required to kill 50% of cells in human SCC-25 squamous carcinoma of the head and neck after various periods of exposure to drugs. Concentrations were derived from the data shown in Fig. 1. Cells were exposed to antifolates in αMEM supplemented with 10% fetal bovine serum

Exposure time (h)	Antifolate concentration (μ*M*) PT523	Methotrexate	Trimetrexate
24	0.54	0.17	1.30
48	0.005	0.026	0.10
72	0.002	0.020	0.013
96	0.00085	0.018	0.005

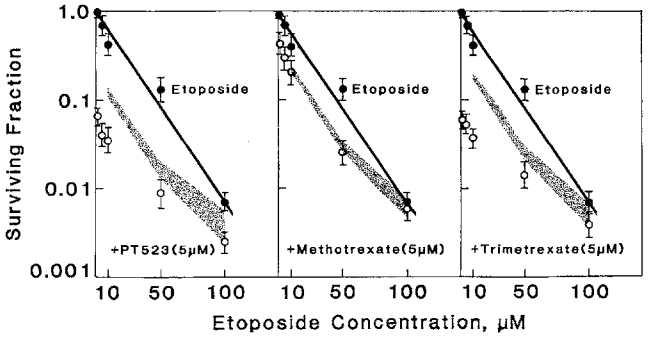

Fig. 2 Survival of human SCC-25 cells (derived from squamous carcinoma of the head and neck) exposed for 2 h to various concentrations of etoposide alone (●) for the last 2 h of a 24-h treatment with an antifolate (5 μ*M*) (○). *Shaded areas* are the envelopes of additivity generated by isobologram analysis. The values are the means of three experiments ± SEM

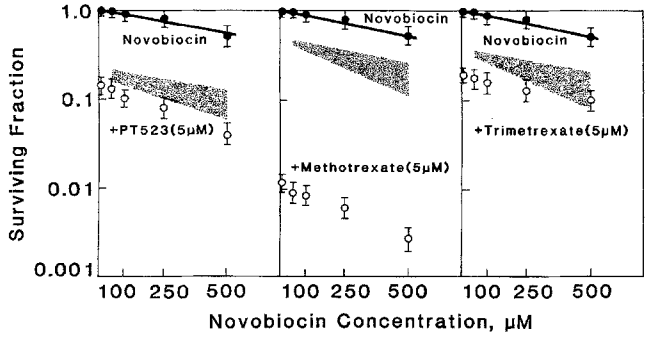

Fig. 3 Survival of human SCC-25 cells (derived from squamous carcinoma of the head and neck) exposed for 2 h to various concentrations of novobiocin alone (●) for the last 2 h of a 24-h treatment with an antifolate (5 μ*M*) (○). *Shaded areas* are the envelopes of additivity generated by isobologram analysis. The values are the means of three experiments ± SEM

antifolate with etoposide killed 1.5–2 logs of SCC-25 cells and produced DNA strand breaks equivalent to 510615 rad. Novobiocin (500 μ*M*, 2 h) killed about 50% of the SCC-25 cells and produced DNA strand breaks equivalent to about 50 rad. PT523 or trimetrexate in combination with novobiocin killed about 1 log of SCC-25 cells and produced DNA strand-

Table 2 Relative DNA strand breaks determined by DNA alkaline elution in human SCC-25 cells (derived from squamous carcinoma of the head and neck) exposed to the antifolates and topoisomerase II inhibitors. Cells were exposed to the antifolate for 24 h with the addition of the topoisomerase II inhibitor during the last 2 h in complete medium supplemented with 10% fetal bovine serum

Antifolate	Rad equivalents[a] Etoposide (50 μ*M*)	Novobiocin (500 μ*M*)
None	155	50
PT523 (5 μ*M*)	510	170
Methotrexate (5 μ*M*)	515	70
Trimetrexate (5 μ*M*)	615	140

[a] The rad equivalent is the radiation dose (rad) required to produce the same level of DNA strand breaking as determined by DNA alkaline elution of the chemotherapy treatment. SCC-25 cells treated with various doses of radiation (100, 200, 300, 400, 500 or 600 rad) were included in the same experiment as the drug treatments. Each experiment was repeated three times

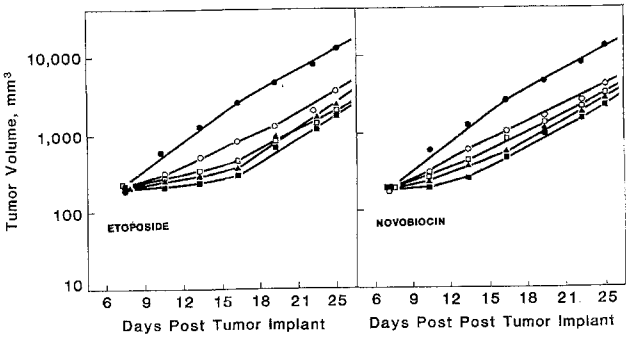

Fig. 4 Growth of the murine SCC-VII squamous carcinoma with no treatment (●), after treatment with antifolate (○), or with the various antifolate/topoisomerase inhibitor combination regimens: PT523 (■), methotrexate (□), trimetrexate (▲)

breaks equivalent to 140–170 rad. However, the SCC-25 cell killing observed with methotrexate and novobiocin was about 2 logs, but only 70 rad equivalent of DNA strand breaks were detected. Therefore cell killing by this combination must result from another mechanism.

The murine SCC VII squamous carcinoma was selected for the in vivo study of these drug combinations because this tumor has been shown to be responsive to antifolates (Fig. 4) [63]. PT523 (0.5 mg/kg) and trimetrexate (2 mg/kg) were administered subcutaneously by continuous infusion over 7 days via an Alzet pump. Trimetrexate was administered by intraperitoneal injection on days 7, 8 and 9. The topoisomerase II inhibitors, etoposide (10 mg/kg) and novobiocin (100 mg/kg), were administered by intraperitoneal injection on days 7, 9 and 11. At the doses used, PT523, methotrexate and trimetrexate gave tumor growth delays of only 2.5, 1.9 and 1.4 days, respectively (Table 3). Etoposide alone produced a tumor growth delay of 2.7 days while combinations of the antifolates with

Table 3 Growth delay of the murine SCC VII squamous carcinoma produced by antifolates and topoisomerase II inhibitors

Treatment group	Tumor growth delay (days)[a]
PT523 (0.5 mg/kg, 7-day infusion)[b]	2.5 ± 0.4
Methotrexate (2 mg/kg, 7-day infusion)	1.9 ± 0.4
Trimetrexate (75 mg/kg, i.p., days 7, 8, 9)	1.4 ± 0.3
Etoposide (10 mg/kg, i.p., days 7, 9, 11)	2.7 ± 0.5
Novobiocin (100 mg/kg, i.p., days 7, 9, 11)	2.0 ± 0.5
PT523/etoposide	8.4 ± 1.0
Methotrexate/etoposide	6.9 ± 0.7
Trimetrexate/etoposide	7.8 ± 0.9
PT523/novobiocin	6.9 ± 0.8
Methotrexate/novobiocin	4.6 ± 0.6
Trimetrexate/novobiocin	4.9 ± 0.5

[a] Tumor growth delay is the difference between the number of days for treated compared with control tumors to reach 500 mm^3. Control tumors reach 500 mm^3 in 12.0 ± 0.4 days.
[b] PT523 and methotrexate were administered via Alzet infusion pumps implanted s.c. on day 6

etoposide were much more effective. The combination of PT523 and etoposide resulted in a tumor growth delay of 8.4 days which was significantly greater than the tumor growth delay produced by methotrexate/etoposide ($P < 0.01$) but not significantly different from the tumor growth delay produced by trimetrexate/etoposide. The combination of PT523 with novofiocin was also the most effective antifolate/novobiocin treatment, producing 6.9 days of tumor growth delay. The combination of PT523 and novobiocin was significantly more effective ($P < 0.01$) than the combinations of methotrexate/novobiocin or trimetrexate/novobiocin. No additional toxicity, as determined by weight loss, was observed with the treatment combinations compared with the individual treatment agents.

Discussion

Elucidation of the mechanism(s) by which specific anticancer cytotoxic agents kills is important because it provides insight into the critical and vulnerable points of cellular metabolism, thus providing for the design of more effective cytotoxic agents and better application of current drugs. The biochemical pathways affected by the antifolates are some of the best understood of all of the anticancer agents. The biochemical effects of the topoisomerase II inhibitors are also well described. However, a knowledge of the direct effects of the drugs on cells did not allow for the pinpointing of the "lethal event" associated with these molecules. Interestingly, it appears that antifolates and topoisomerase II inhibitors as well as other diverse cytotoxic agents create a critical imbalance in cellular metabolism that triggers the common pathway of death through apoptosis, a normal process for cell turnover or elimination [65].

As early as 1986, Zwelling et al. [71] reported that exposure of L1210 murine leukemia cells in culture to antimetabolites (ara-C, hydroxyurea or 5-azacytidine) can augment the cytotoxicity and DNA strand-break frequency of topoisomerase II inhibitors. Studying the human histiocytic lymphoma cell line U937, Lorico et al. [36] found that exposure of the cells to a low concentration of methotrexate for 16 h prior to exposure to etoposide results in increased cytotoxicity of the etoposide and increased DNA strand breakage. These findings have been extended to human ovarian SW626 carcinoma cells in culture with similar positive findings. Cell cycle studies have indicated that initially exposing the cells to methotrexate leads to synchronization of the population so that most of the cells are in S-phase when topoisomerase II levels are the highest at the time of etoposide treatment, thus providing a maximal number of targets for the drug [18, 36]. Fry [21] found that exposure of L1210 cells to the "nonclassical" antifolate trimetrexate prior to treatment with etoposide produces a similar potentiation of the cytotoxicity of the drug and speculated that depletion of intracellular ATP by the antifolate may be responsible for this effect. We showed that the new antifolate PT523 was as effective or more effective than methotrexate or trimetrexate in producing synergistic killing of human SCC-25 squamous carcinoma cells in culture in combination with etoposide or novobiocin, and that this increased cytotoxicity generally correlated with increased DNA strand breaks (except for the combination of methotrexate and novobiocin which caused greater than expected cell killing).

The antifolate/topoisomerase II combinations were also very effective in vivo against the murine SCC VII squamous carcinoma. The treatment regimens that included PT523 were most effective and PT523/etoposide was the most effective therapy in this study.

Mechanistically, perhaps partial inhibition of one critical biochemical pathway with an antifolate and

partial inhibition of a second critical biochemical pathway with a topoisomerase II inhibitor results in the triggering of apoptosis in cells that may otherwise survive.

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