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# Synergistic effect of inhibitors of topoisomerase I and II on chromosome damage and cell killing in cultured Chinese hamster ovary cells

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**Abstract.** Simultaneous treatment of cultured Chinese hamster ovary cells with the topoisomerase I inhibitor camptothecin and the topoisomerase II inhibitor 4′-(9-acridinylamino)-methanesulfon-*m*-anisidide results in a clear synergistic effect on both chromosome damage detected at metaphase and loss of colony-forming ability. In contrast, the effect of combined treatment with these topoisomerase inhibitors on sister chromatid exchanges was not significantly different from that expected if the effects were additive. Taken as a whole, these results seem to support the hypothesis that topoisomerase inhibitors can lead to cell death, presumably when DNA replication forks collide with drug-stabilized cleavable complexes. Nevertheless, no evidence of apoptosis was obtained from DNA fragmentation analysis. The possible clinical implications of our findings are discussed.

**Key words:** Topoisomerase inhibitors – Chromosome damage – Sister chromatid exchanges – Synergism

## Introduction

DNA topoisomerases are enzymes dealing with topological changes in the DNA molecule that take place during DNA metabolism (e.g., replication, transcription, segregation of daughter molecules, repair recombination) through concerted breakage and reunion of the molecule. Eukaryotic DNA topoisomerase II is the target of a number of potent anticancer drugs of different chemical nature, and topoisomerase II inhibitors are well established in tumor chemotherapy [25, 30]. In contrast in spite of the activity demonstrated for the topoisomerase I inhibitor camptothecin against a number of experimental neoplasms [11] as well as in xenograft models [12], it shows an unacceptable toxicity profile [27], and the activity of its derivative topotecan [10-hydroxy-9-dimethylaminomethyl-(*S*)-camptothecin;SK&F 104864] appears to be limited by neutropenia [31].

It is now widely accepted that topoisomerase poisons inhibit the enzyme reaction by stabilizing an abortive reaction intermediate, the "cleavable complex," whereby the DNA is cleaved but cannot be readily resealed. With regard to cell killing, there is good evidence that a collision of moving replication forks with drug-stabilized topoisomerase-DNA cleavable complexes converts the normally reversible complexes into lethal lesions [16, 18]. We have recently reported on the importance of replication-fork progression for the induction of both chromosomal aberrations and sister chromatid exchanges (SCE) by topoisomerase inhibitors [6], presumably when replication forks collide with cleavable complexes and cannot progress any further.

It has been proposed that such a block of replication forks by stabilized cleavable complexes would induce the pathway leading to DNA degradation and, perhaps, cell death [19], for it has been reported that a variety of conditions producing the arrest of replication lead to irreversible DNA fragmentation [17, 21].

Selective killing of tumor cells in association with a minor effect on normal proliferative or non-proliferative cells is the main goal of cancer therapy. On the other hand, any protocol that would result in an enhancement of the effect of drugs that are highly toxic to noncancerous cells, thus avoiding the use of high concentrations, should be of clinical interest. For example, we have recently shown that the effectiveness of the radiomimetic agent bleomycin can be potentiated by posttreatment administration of the polycationic compound poly-D-lysine [4].

In the present investigation we analyzed the effectiveness of combined treatment with inhibitors of topoisomerases I and II [camptothecin and 4′-(9-acridiylamino)-methanesulfon-*m*-anisidide (m-AMSA), respectively) for the induction of chromosomal aberrations, SCE, and cell killing in cultured Chinese hamster ovary (CHO) cells. Our

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**Table 1.** Cytogenetic effects of single and combined treatment with inhibitors of topoisomerase I and II. A total of 100 metaphases were scored for chromosomal aberrations and SCE (*Topo* Topoisomerase)

| Inhibitors of | | Abnormal metaphases | Deletions | Exchanges | Total scorable aberrations | SCE ± SE |
|---|---|---|---|---|---|---|
| Topo I CPT | Topo II m-AMSA | | | | | |
| – | – | 3 | – | 3 | 3 | 5.82±0.66 |
| 2.5 μ*M* | – | 21 | 5 | 17 | 22 | 28.56±1.48 |
| – | 0.25 μ*M* | 17 | 10 | 16 | 26 | 19.06±1.21 |
| 2.5 μ*M* | 0.25 μ*M* | 73 (14)[a] | 43 | 54 | 97 (2.02)[b] | 40.24±1.75 |
| 5.0 μ*M* | – | 24 | 10 | 27 | 37 | 26.06±1.41 |
| – | 0.50 μ*M* | 22 (1)[a] | 4 | 26 | 30 | 21.20±1.27 |
| 5.0 μ*M* | 0.50 μ*M* | 64 (5)[a] | 27 | 79 | 106 (1.58)[b] | 36.38±1.67 |

[a] Multiple aberrant metaphases are given in parentheses

[b] The synergism factor is given in parentheses. Note that these figures must have been underestimated, taking into account the number of cells showing multiple aberrations following the combined treatment

results show a high degree of synergism with regard to chromosome damage and proliferative death, whereas the effect was simply additive in terms of SCE. The possible implications of these results for a better knowledge of the mechanisms of cell death as a consequence of inhibition of topoisomerases are discussed.

## Materials and methods

*Materials.* 5-Bromodeoxyuridine (BrdU), fluorodeoxyuridine (FdU), deoxythymidine (dT), deoxycytidine (dC) and the topoisomerase I inhibitor camptothecin (CPT) were purchased from Sigma Chemical Co. (St. Louis, Mo., USA), and m-AMSA (NSC-249992) was obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, Md., USA).

*Cell culture.* CHO6 cells were cultured as monolayers in McCoy's 5A medium supplemented with 10% fetal bovine serum, 2 m*M* L-glutamine, and the antibiotics penicillin (50 units/ml) and streptomycin (50 μg/ml). Cells were grown in a dark environment at 37° C in an atmosphere containing 5% $CO_2$.

*Treatment of cells for chromosome studies.* For labeling of cells with BrdU, they were first grown for 18 h in the presence of 5 μ*M* BrdU, 5 μ*M* dT, 100 μ*M* dC, and 1 μ*M* FdU to suppress endogenous dT synthesis [29] and then treated for 30 min with either CPT, m-AMSA, or a combination of both at different doses. By this procedure, the cells are exposed to the topoisomerase inhibitors while they are in their second S phase of incorporation of BrdU into the DNA [5]. After a thorough washing, cells were grown for an additional 6-h period in the same mixture of nucleosides given before and were then Colcemid-treated for the last 2 h of treatment. The cells were then collected by mitotic shakeoff, treated with hypotonic 0.075 *M* KCl (2–3 min) at 37° C, and finally fixed in two washes with methanol-acetic acid (3:1, v/v). Standard cytological preparations were then made and either stained in 3% Giemsa or processed by a modified fluorescence plus Giemsa (FPG) technique [3] for the scoring of chromosomal aberrations and SCE, respectively.

The experiments were carried out at least twice. To quantify the possible interaction between topoisomerase inhibitors we used a synergism factor (SF), defined as the ratio of the effect of the combined treatment to the sum of the effects of the two inhibitors given separately [24]. We regard an SF of 1.5 or more as indicating synergism between the inhibitors.

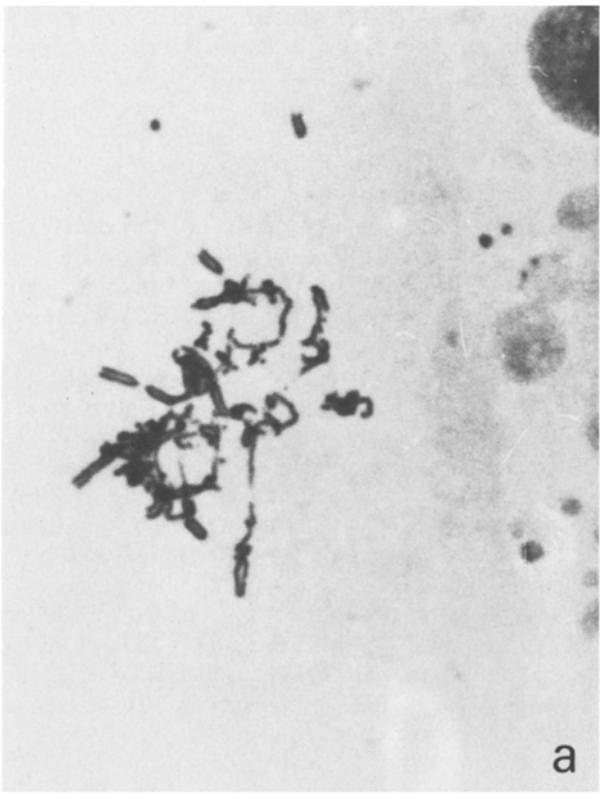
*Clonogenic survival.* Trypsinized CHO cells were seeded at a density of 200 cells/60-mm dish. Triplicate cultures were established for each treatment condition. After the cells had been allowed to attach, either they were left untreated (controls) or 1 μ*M* CPT, 0.1 μ*M* m-AMSA, or a combination of both topoisomerase inhibitors were placed for 3 h in the culture medium, after which the dishes were washed with phosphate-buffered saline (PBS) and then incubated for 7 days at 37° C in a humidified atmosphere. Finally, the cells were fixed with methanol, stained in 10% Giemsa solution, and dried, and the number of colonies that had formed were counted.

*Analysis of DNA fragmentation by agarose gel electrophoresis.* DNA was extracted and separated following standard procedures, basically as described by Bertrand et al. [1] for Chinese hamster fibroblasts. Briefly, cells were treated for 3 h with topoisomerase inhibitors, and 24 h later they were scraped into their culture medium, harvested by centrifugation at 800 rpm for 6 min, and washed twice with ice-cold PBS. For extraction of DNA by a salting-out procedure, cells were incubated for 16 h at 48° C in 0.5 *M* TRIS (pH 8.0) containing 20 m*M* ethylenediaminetetraacetic acid (EDTA), 10 m*M* NaCl, 1% sodium dodecyl sulfate (SDS), and 0.5 mg proteinase K/ml. Following incubation, the salt concentration (NaCl) was raised to 1 *M* and the tubes were shaken vigorously. Samples were centrifuged (30 min, 12,500 rpm), supernatants were collected, and DNA was precipitated. DNA electrophoresis was performed for 1 h at 7 V/cm followed by 3 h at 2 V/cm in 1.2% agarose gel in TRIS-borate buffer (pH 8.0). The gel was stained with 0.2 μg ethidium bromide/ml for 30 min, washed, and transilluminated with UV light for photography.

## Results

### *Effect of single and combined treatments with CPT and m-AMSA on chromosome damage and SCE*

Table 1 shows the effect of single treatments with CPT (2.5 and 5 μ*M*) or m-AMSA (0.25 and 0.5 μ*M*) on chromosomal aberrations and SCE following their application for 30 min either alone or in combination during the second S-period of BrdU incorporation into DNA in CHO cells. As can be seen, both topoisomerase inhibitors were capable of efficiently inducing chromosome damage as well as SCE (Fig. 1), in agreement with the findings previously reported by us and other investigators [5, 6, 10, 28].



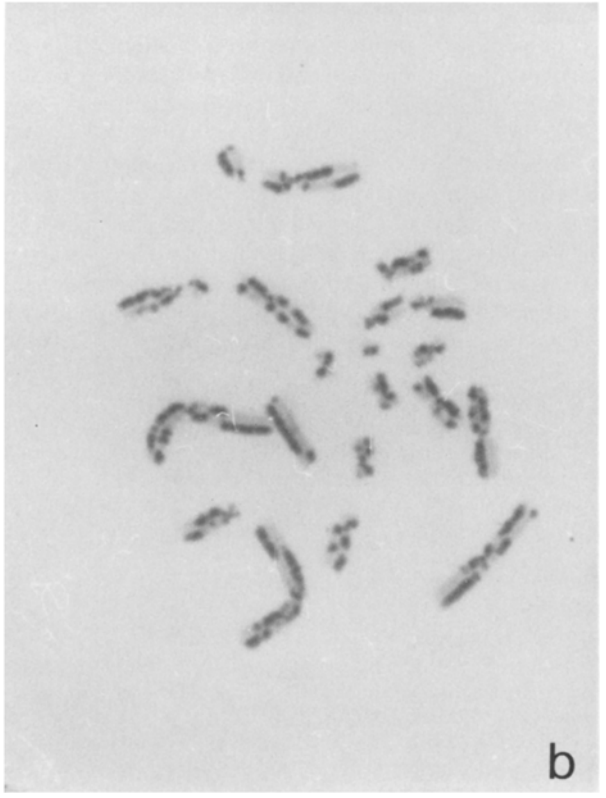




**Fig. 1 a and b.** Cytogenetic damage observed after combined treatment with 0.5 μ*M* m-AMSA and 5 μ*M* CPT. **a** Heavily damaged metaphase. **b** High frequency of SCE

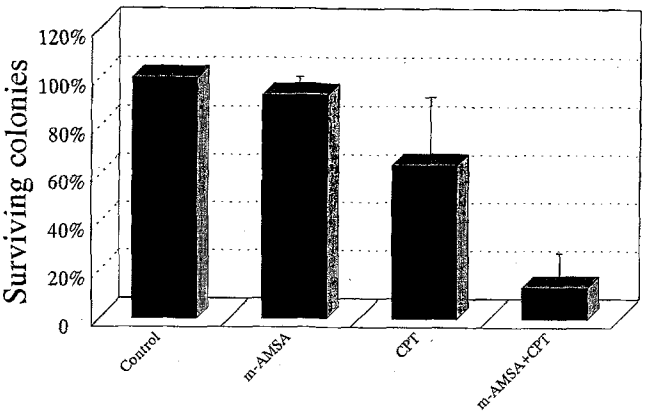



**Fig. 2.** Effect of treatment with 0.1 μ*M* m-AMSA, 1 μ*M* CPT, or a combination of both on the clonogenic survival of CHO cells. Each value represents the mean number of colonies scored in triplicate. Bars represent standard errors of the mean. As can be seen, simultaneous treatment with both topoisomerase inhibitors resulted in a clear, synergistically negative effect on clonogenic survival ($P < 0.001$; chi-squared test)

Treatment of cells with both inhibitors simultaneously, on the other hand, resulted in a clear synergistic effect on chromosome damage, as reflected by the total number of abnormal metaphases observed as well as the yield of scorable aberrations (Table 1). Cells showing multiple aberrations were also observed after the combined treatments. Contrasting with the enhancement of chromosome damage detected when both CPT and m-AMSA were given together, the effect on the frequency of SCE was in the range of additivity for both doses assayed (Table 1).

*Effect on clonogenic survival*

The existence of a close relationship between gross chromosome damage and reproductive cell death has been well established [9, 20]. We also studied the colony-forming ability of cells treated with CPT, m-AMSA, or both in combination. Figure 2 shows that 0.1 μ*M* m-AMSA and 1 μ*M* CPT had a negative effect on the colony-forming ability of treated cells as compared with control cultures, which was more evident at this chosen dose of CPT. As can be seen from this figure, in good agreement with the result previously obtained for chromosome damage, the combined treatment with both topoisomerase poisons resulted in a clear synergistic effect, with the loss of clonogenic survival being more than twice that expected if the effects were simply additive (Fig. 2).

*Effect on DNA fragmentation*

To test whether the cytotoxicity observed as a loss of clonogenic survival could be attributable to a typical mechanism of programmed cell death (apoptosis) as a consequence of topoisomerase inhibition [15, 22], we analyzed agarose gels for visual evidence of endonuclease activation. As can be seen in Fig. 3, DNA fragmentation as a con-

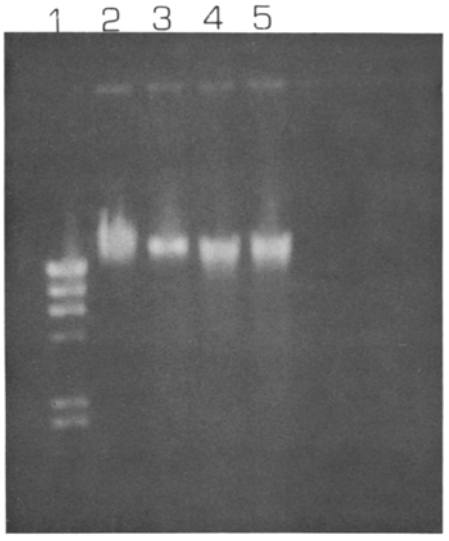



**Fig. 3.** DNA cleavage induced by inhibitors of topoisomerase I and II. Treated cells were given a 3-h treatment with 1 μ*M* CPT (*lane 3*), 0.1 μ*M* m-AMSA (*lane 4*), or a combination of both (*lane 5*), and DNA was extracted after a recovery period of 24 h (*Lane 1* Lambda-*Hind*III molecular-weight marker, *lane 2* untreated control CHO6 cells)

sequence of treatment with either CPT alone, m-AMSA alone, or a combination of both was visible. Nevertheless, no evidence was obtained of the internucleosomal DNA cleavage (internucleosomal ladder) typically observed after endogenous endonuclease attack.

## Discussion

There is a growing body of evidence that the ultimate efficacy of antitumor treatment may be associated with the ability of tumor cells to respond by the programmed-cell-death process known as apoptosis. The possible modulation of cell response by apoptosis has opened a new field in antitumor strategies in recent years [13, 14]. Both topoisomerase I and II inhibitors can trigger apoptosis [8, 15, 22], which results in extensive DNA strand breakage as a consequence of activation of endogenous endonucleases. Preferential apoptosis of S-phase cells has been observed following treatment of HL-60 cells with both CPT and m-AMSA [14].

Collision of DNA replication forks with cleavable complexes has been proposed as a mechanism inducing cell death [18], and the results we obtained on the importance of replication-fork progression for the induction of DNA double-strand breakage and chromosomal aberrations by inhibitors of topoisomerases [6] seem to support such a hypothesis.

The present investigation shows that simultaneous treatment with both CPT and m-AMSA results in a strong synergistic effect on chromosome damage and cytotoxicity as compared with the effect of topoisomerase inhibitors given alone. In our opinion, these observations suggest that topoisomerase I and II inhibitors owe at least some of their cytotoxicity to their genotoxic effects.

Taudou et al. [32] found that when stimulated splenocytes were subjected to the concerted action of topoisomerase I and II poisons (CPT and etoposide), the outcome was extensive DNA degradation to nucleosomal size resulting from secondary events (apoptosis). Nevertheless, according to other reports, the cytotoxicity of topoisomerase II-targeting compounds is reduced in the presence of CPT [7, 23]. An explanation for these conflicting data is not at hand, but our results seem to support the findings of Taudou et al. [32] in that the concerted action of topoisomerase I and II inhibitors can result in a clear, synergistically cytotoxic effect.

Synergistic cell killing by ionizing radiation and the topoisomerase I inhibitor topotecan has recently been reported [26], and these results have been interpreted as supporting a possible role of the enzyme in the repair of some form of damage induced by ionizing radiation [2, 26]; however, in our opinion, an alternative explanation based on the possible triggering of the programmed-cell-death process also seems likely and cannot be ruled out.

The observation that the effect of combined treatment with CPT and m-AMSA on SCE was simply additive seems to support the hypothesis that the synergistic effect on chromosome damage and subsequent cytotoxicity cannot be attributed to the direct action of CPT and m-AMSA on DNA but that secondary DNA damage must be involved. Although DNA topoisomerase I and II could substitute for each other in a number of their metabolic functions [25], the simultaneous inhibition of both enzymes could trigger the process of cell death. Nevertheless, although DNA cleavage after topoisomerase inhibition was evident, we did not find the typical internucleosomal pattern of DNA fragmentation that characterizes apoptosis.

Regardless of the exact molecular mechanism(s) leading to the programmed-cell-death process known as apoptosis, which deserves further investigation, the present results as well as those reported from other laboratories [19, 32] seem to have possible clinical implications for an improvement in the chemotherapy of tumors by combined treatment with low doses of topoisomerase inhibitors.

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