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Effects of ICRF-193, a catalytic inhibitor of DNA topoisomerase II, on sister chromatid exchange

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To investigate whether mammalian DNA topoisomerase II is directly involved in recombination events, the effects of ICRF-193, a specific catalytic inhibitor on sister chromatid exchange (SCE), were examined in MR-6 cells. ICRF-193 only slightly elevated SCE formation after 3 or 44 h treatments, while VP-16, a cleavable complex forming type of topoisomerase II inhibitor, caused significant enhancement. ICRF-193 had no effect on *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-induced SCE formation. It would thus appear that the inhibition of topoisomerase II does not affect recombinational repair, and that topoisomerase II inhibitors such as VP-16 and 4'-(9-acridinyl amino) methane sulfon-*m*-anisidide induce SCE through production of DNA strand breaks, rather than by inhibiting the enzyme activity.

Key words: ICRF-193, sister chromatid exchange, topoisomerase II inhibitor.

Introduction

DNA topoisomerases I and II catalyze relaxation of supercoiled DNA by transiently introducing single- and double-strand breaks, respectively. In DNA replication and transcription, these enzymes are involved in resolving torsional strains of DNA which accumulate during the macromolecule synthesis.[1] Topoisomerase II, but not topoisomerase I, can unlink multiply intertwined DNA rings, and plays an important role in mitotic chromosome dynamics such as condensation and segregation.[1]

There is evidence that topoisomerase II is also involved in recombination: in higher eukaryotes, it appears to suppress this process and in budding yeast, topoisomerase II temperature-sensitive mutants have a high frequency of mitotic recombination in the rDNA at a semi-permissive temperature.[2] Treatment of yeast with 4'-(9-acridinyl amino) methane sulfon-*m*-anisidide (*m*-AMSA), a topoisomerase II inhibitor, increases gene conversion and reciprocal exchange.[3] In mammalian calls, inhibitors of topoisomerase II like VM-26 or m-AMSA increase quadriradial chromosome formation, due to intermediate reciprocal exchange of chromatids from different chromosomes, and integration of SV40 DNA or adeno-associated virus vectors into host genome.[4,5] Since *m*-AMSA and VM-26 are cleavable complex stabilizing type drugs,[6] they may exert their biological effects through the inhibition of topoisomerase II activity or topoisomerase II-mediated DNA cleavage. We have identified a separate group of non-cleavable stabilizing topoisomerase II inhibitors called bisdioxopiperazines.[7,8] One of these, ICRF-193 was recently shown to inhibit topoisomerase II catalytic activity, but not to stabilize the cleavable complex.[8] Thus, the drug has advantages over other topoisomerase II inhibitors for investigating the enzyme's functions.

In the present study, to answer the question of whether topoisomerase II plays a direct role in DNA recombination, we examined the effect of ICRF-193 on SCE formation, which reflects recombination between two homologous DNA duplexes.

Materials and methods

Cells and culture conditions

MR-6,[9] a Hela S3 strain was cultured at 37°C in Dulbecco's modified Eagle's medium, supplemented with 10% calf serum, penicillin (10 units/ml) and streptomycin (100 μg/ml) in a humidified atmosphere of 5% CO_2 in air.

Drugs

N-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) was purchased from Sigma (St Louis, MO). ICRF-193 and

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VP-16 were obtained from Zenyaku Kogyo (Tokyo, Japan) and Bristol-Meyer Squibb (Brea, CA), respectively. The carcinogen and the two drugs were dissolved in dimethyl sulfoxide and directly added to the culture medium. The other reagents used were 5′-bromo-2-deoxyuridine (BrdU) (Boehringer Mannheim, Germany), Hoechst 33258 (Sigma), demecolcine (Colcemid) (Sigma) and Giemsa (E Merck, Darmstadt, Germany). Stock solutions of MNNG (10 mM), VP-16 (10 mM) and ICRF-193 (10 mM) were kept frozen at −20°C and thawed immediately before use.

SCE analysis

Exponentially growing MR-6 cells (1×10^5) were treated with various concentrations of the test agents for the periods indicated in the figure legends, washed with growth medium and grown for two cell cycles in the presence of BrdU (final concentration of 5 μM). Colcemid (final concentration of 0.02 μg/ml) was added for 2 h before harvesting the cells. Cells were kept in the dark under cover. Differential staining for SCE was performed by a modification of the method of Perry and Wolff.[10] Samples on slides, prepared by routine air-drying methods, were stained with 0.025 μg/ml of Hoechst 33258 for 20 min, rinsed in deionized water and mounted in 1/30 M Sörensen buffer solution (pH 7.4), under cover slips. The slides were then simultaneously heated at 65°C on a slide warmer and exposed to Black Ray long wave UV at a distance of 6 cm for 30 min. After removal of the cover slips, the samples were rinsed briefly with deionized water, and washed in 2 × SSC (0.3 M sodium chloride–0.03 M tri-sodium citrate). They were then rinsed throughly with deionized water and stained with 2% giemsa solution (1:50 in 1/30 M Sörensen buffer solution, pH 7.4) for 30 min. SCE frequency was determined for 20 metaphases of control and drug-treated cells and expressed as SCE per chromosome.

Results

Effects of ICRF-193 on SCE induction in MR-6 cells

To determine if ICRF-193 can induce significant numbers of SCE, we first examined the effects of increasing concentrations of the drug on growth of MR-6 cells. MR-6 cells were exposed to various concentrations of ICRF-193 and cultured for 4 days. MR-6 cell growth was reduced by ICRF-193 to 78% by 0.1 μM amd 35% by 0.3 μM, respectively (Figure 1). No effects were observed with a 1 h treatment at these doses. SCE induction was next examined under various conditions. When MR-6 cells were treated with various concentrations of ICRF-193 for 3 h from 0 to 3 h or 22 to 25 h after the addition of BrdU, SCE formation increased only slightly as compared to the control case (Figure 2A). Continual exposure of cells to ICRF-193 for 44 h resulted in a slightly increased SCE formation (Figure 2B). At above 0.3 μM, ICRF-193 induced abnormally condensed or entangled chromosomes[7] and thus SCE induction could not be examined.

Effects of VP-16 on SCE induction

VP-16 has been used as an inducer of SCE.[11,12] Since it is also a cleavable complex stabilizing topoisomerase II inhibitor,[13] the drug might induce

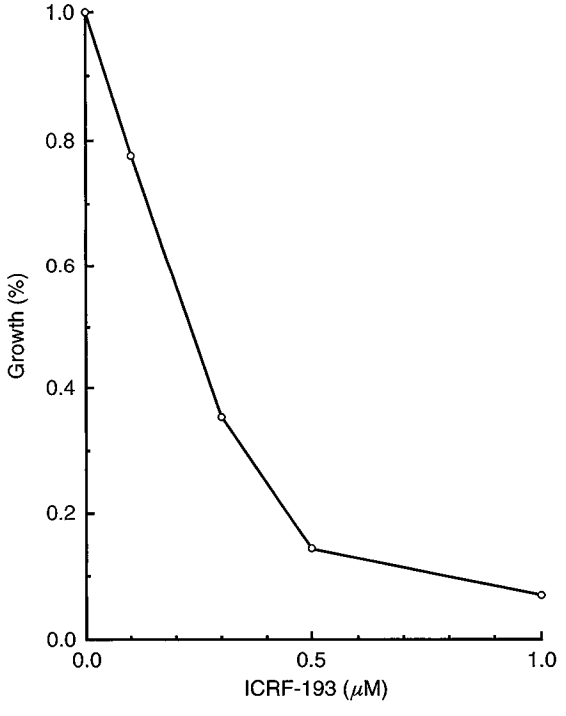

Figure 1. Effects of ICRF-193 on growth of MR-6 cells. MR-6 cells were seeded at 3×10^4 cells/60 mm dish, and from the next day, treated with various concentrations of ICRF-193 for 4 days. Cell numbers were then counted using Coulter Industrial D cell counter (Coulter Electronics, UK), and percent growth expressed as percentages of control cell value.

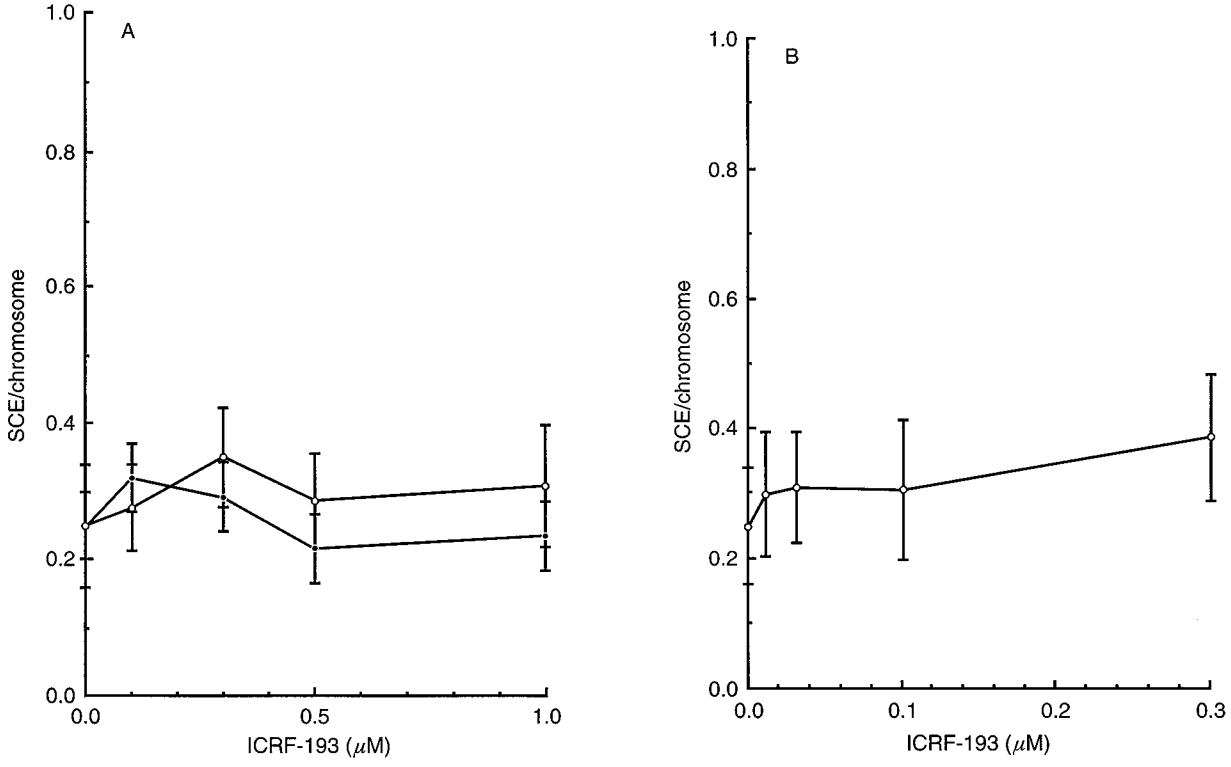

Figure 2. SCE formation after 3 or 44 h treatments with ICRF-193. MR-6 cells were exposed to various concentrations of ICRF-193 from 0 to 3 h (●) or 22 to 25 h (○) after addition of BrdU (A) or for 44 h (B).

SCE through inhibition of topoisomerase II activity or topoisomerase II-mediated DNA cleavage. Therefore, as a control for ICRF-193, VP-16 was tested under the same experimental conditions. When MR-6 cells were exposed to various concentrations of VP-16 for the first 3 h (0 to 3 h) after addition of BrdU, SCE induction was significantly increased (Figure 3A). Similar results were obtained with a 3 h exposure of cells to VP-16 from 22 h after the addition of BrdU.

A much greater, clearly dose-dependent induction of SCE was observed when cells were continually exposed to low concentrations of VP-16 (Figure 3B). Thus VP-16 is high inducer of SCE, compared with ICRF-193.

Effects of ICRF-193 on MNNG-induced SCE

If topoisomerase II is involved in SCE formation, the reported induction by MNNG[14] should be reduced in the presence of ICRF-193. MNNG indeed induced a high frequency of SCE (Figure 4), but neither 3 nor 44 h treatments with ICRF-193 exerted any significant effects.

Discussion

SCE formation, a sensitive indicator of DNA damage and mutagenesis,[15] is induced in S phase and is representative of recombinational repair.[16–18] The present work provided evidence that ICRF-193 itself does not induce SCE and that it does not inhibit MNNG induction of SCE. This clearly contrasted with findings for VP-16, a cleavable complex forming type inhibitor.[11,12] Since ICRF-193 is a catalytic inhibitor, these results suggest that VP-16-induced SCE formation is in fact caused by topoisomerase II-meditated DNA breaks and not by inhibition of the enzyme activity. The different effects of ICRF-193 and VP-16 on SCE formation are consistent with the concept that replication stops at lesions created by various DNA damaging agents and SCE occurs at blocked replication forks, since VP-16, but not ICRF-193 has been shown to inhibit DNA chain elongation.[19] Distinct biological effects of ICRF-193

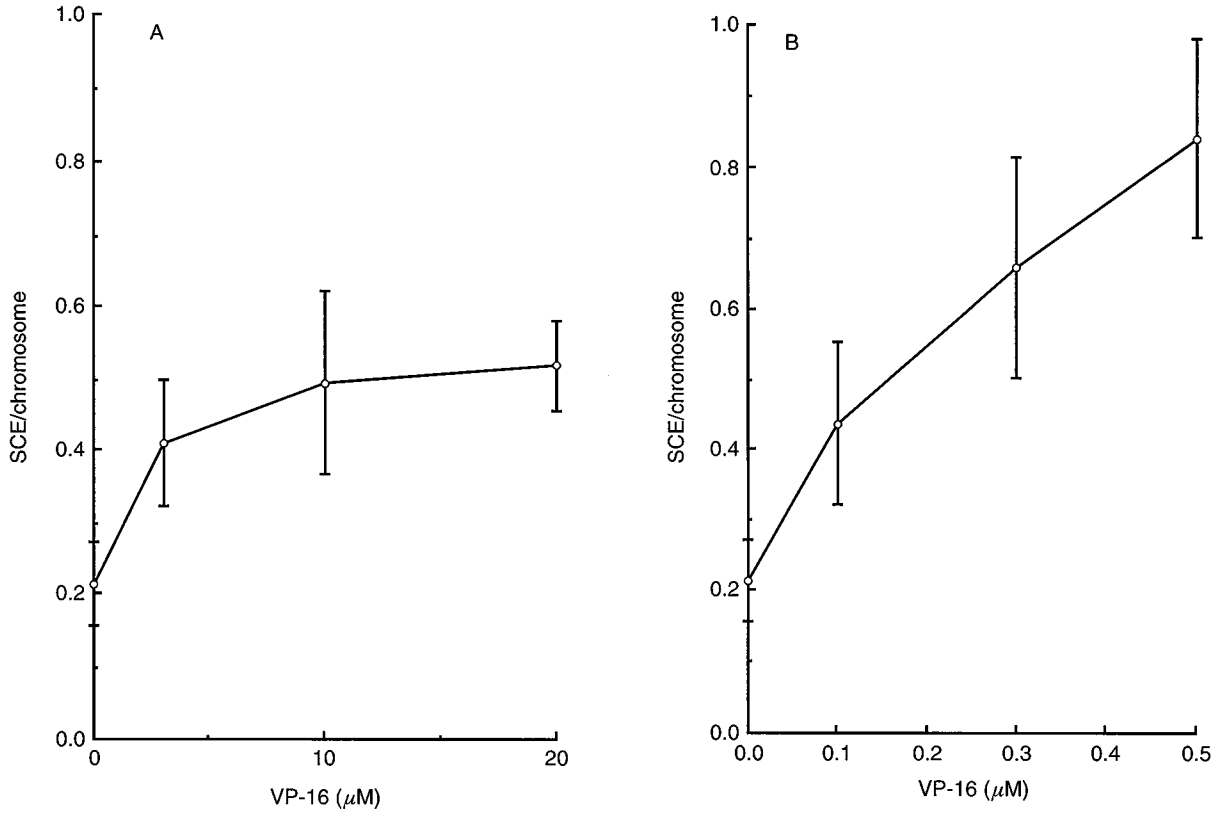

Figure 3. VP-16-induced SCE after 3 or 44 h treatments with VP-16. Cells were exposed to various concentrations of VP-16 from 0 to 3 h after addition of BrdU (A) or for 44 h (B).

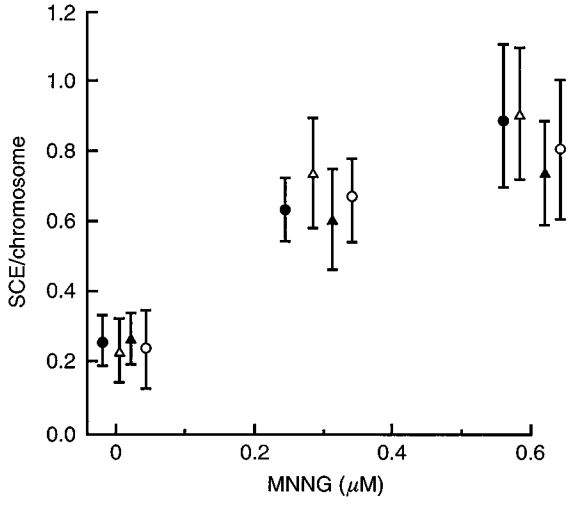

Figure 4. Effects of ICRF-193 on MNNG-induced SCE formation. MR-6 cells were pretreated with 0.3 or 0.6 μM MNNG for 1 h. After removing the drug, the cells were exposed to no drug (●) or 1 μM ICRF-193 from 0 to 3 h (△) or 22 to 25 h (▲) or to 0.03 μM ICRF-193 for 44 h (○) in the presence of BrdU.

and VP-16 were also observed on DNA synthesis and cell cycle progression.[20–22]

In yeast, two types of sister chromatid recombinations are presumed to occur:[23] one is S-phase associated and the other is S-phase independent. To account for the former, observed in mammalian cells, it is presumed that DNA polymerase stops at damaged regions but synthesis along the undamaged template continues with subsequent replacement of the unreplicated region by recombination with its homolog on the complete strand.[24] In the majority of cases gene conversions result. The latter is associated with excision repair creating recombinogenic lesions, and causes gene conversions and reciprocal recombination in yeast.[23] Aratani *et al.*[25] showed both homologous and non-homologous recombinations to be enhanced by treatment with ICRF-193 in mammalian cells, as determined using a plasmid harboring the *aprt* gene. Their findings are in contrast with our present data that ICRF-193 does not enhance SCE. However, this discrepancy might be explained by the presence of two types of

recombination noted in the yeast system, i.e. SCE is induced in a replication-dependent manner while integration of plasmid into host cells and recombination between plasmids may be replication independent. The idea is supported by the observation that VM-26 and *m*-AMSA induce quadriradial chromosomes which are intermediates in reciprocal exchange of duplex DNA replication,[26] and that Adeno-associated virus vectors integrate into the host genome in all cell cycle stages.[5]

Topoisomerase II has been implicated in many aspects of DNA metabolism such as DNA replication, trascription and chromosome dynamics in the M phase.[1] There are contradictory data on participation of topoisomerase in recombination in higher eukaryotes and *Escherichia coli*. In budding yeast, a null topoisomerase I strain, a topoisomerase III mutant and topoisomerase II temperature-sensitive mutant at the semi-permissive temperature all exhibit a high frequency of mitotic recombination in the rDNA cluster, showing that topoisomerases suppress recombination.[2] On the other hand, in *E. coli*, type II DNA topoisomerase promotes illegitimate recombination.[27] Our data indicate a difference from both these cases, since ICRF-193 did not stimulate SCE and did not affect MNNG-induced SCE, thus suggesting that mammalian topoisomerase II is not involved in recombination.

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