Inhibitors of topoisomerases do not block the passage of human lymphocyte chromosomes through mitosis

A. T. SUMNER

MRC Human Genetics Unit, Western General Hospital, Crewe Road, Edinburgh EH4 2XU, UK

Summary

Cultured human lymphocytes have been treated with a number of topoisomerase inhibitors, to see whether topoisomerase II is involved in the process of chromosome segregation at anaphase. Results were assessed by examination of cytogenetical preparations of spread chromosomes. Four effects were observed, although no inhibitor produced all four effects. These effects were: inhibition of entry into mitosis; chromosome breakage and rearrangement; inhibition of chromosome condensation; and inhibition of chromosome segregation. Evidence for the last was ambiguous. Although there was evidence that separation of chromatids was affected when cells were treated with colchicine as well as topoisomerase II inhibitors (most notably with nalidixic acid, which resulted in complete fusion of the chromatids), no evidence was obtained to show that, in the absence of colchicine, cells treated with inhibitors could not proceed through anaphase normally. The topoisomerase I inhibitor, camptothecin, differed from the topoisomerase II inhibitors in not showing any effect on chromosome condensation or any significant effect on segregation.

Key words: chromosome condensation, chromosome damage, chromosome segregation, mitosis, topoisomerases.

Introduction

It is to be expected that the enzyme topoisomerase II would be involved in mammalian chromosome segregation, both on theoretical grounds, and by analogy with the situation in lower eukaryotes. It is believed that the daughter DNA molecules resulting from replication are catenated, and therefore require the action of topoisomerase II to disentangle them (Hsieh and Brutlag, 1980; Sundin and Varshavsky, 1981; Murray and Szostak, 1985). Extensive experiments with yeast topoisomerase II mutants indicate that the enzyme is needed for disjunction of chromosomes, both at mitosis (di Nardo et al. 1984; Uemura and Yanagida, 1986; Uemura et al. 1987; Holm et al. 1989) and at meiosis (Rose et al. 1990). However, results from experiments on yeasts may not necessarily be applicable to higher organisms, and there is no clear evidence yet that topoisomerase II is involved in chromosome segregation in mammalian cells. In any case, the chromosomes of yeasts are too small for studying some of the processes of segregation easily.

In the present study, the possible involvement of topoisomerase II in mammalian chromosome segregation has been investigated using a number of inhibitors of topoisomerase II, and studying the effects of these on chromosomes at the different stages of mitosis. It was reasoned that if chromosome segregation were prevented by inhibition of topoisomerase II, an accumulation of chromosomes at the metaphase stage of mitosis might be expected; as described by Uemura and Yanagida (1986) for fission yeast, the mitotic spindle would pull but fail to separate chromosomes. Inhibitors of topoisomerase II are used extensively in cancer chemotherapy (D'Arpa and Liu, 1989), and their effects on cells have therefore been studied extensively; their main effects on the cell cycle appear to be blockage in interphase, induction of chromosome damage, and inhibition of chromosome condensation (Huang et al. 1973; Pommier et al. 1988; Rowley and Kort, 1989), but an unequivocal effect on chromosome segregation has not yet been reported.

Experiments in which mammalian cells have been treated with topoisomerase II inhibitors (Charron and Hancock, 1991; Downes et al. 1991) may be inconclusive, because many of these inhibitors cause extensive chromosome damage which may itself, independently of an action on topoisomerase II, lead to an appearance of mal segregation. In the present experiments, I have concentrated on studying the behaviour of the chromosomes during mitosis, rather than relying on signs of non-disjunction, such as micronuclei, and strands of chromatin connecting daughter nuclei, which could arise from a variety of causes.

Materials and methods

Human peripheral blood lymphocyte cultures were grown...
cultures had colchicine (0.5 μg/5 ml culture) added at the same time as the inhibitor; the other set of cultures did not have colchicine added. The topoisomerase inhibitors used are listed in Table 1. Control cultures had no inhibitor added, but if the inhibitor was dissolved in dimethyl sulphoxide (DMSO), all the cultures in that experiment, including the control, had the same amount of DMSO added (including the vehicle for the inhibitor), as some results appeared to indicate that DMSO alone might influence the proportions of cells at different mitotic stages. Inhibitors would prevent normal segregation of human chromosomes. This question was tackled in two ways. In cultures lacking colchicine, it was expected that, if the inhibitor was dissolved in dimethyl sulphoxide (DMSO), all the cultures in that experiment, including the control, had the same amount of DMSO added (including the vehicle for the inhibitor), as some results appeared to indicate that DMSO alone might influence the proportions of cells at different mitotic stages. At the end of the culture period, the cultures were treated with hypotonic solution (0.075 M potassium chloride), fixed in methanol/acetic acid followed by spreading with colchicine. This may well have been because fixation in methanol/acetic acid followed by spreading on slides disrupts the cell membrane and dissolves the cytoplasm, and the two daughter telophase nuclei could therefore become widely separated from each other. The proportions of mitotic cells in the different stages of mitosis are shown in Figs 2, 3 and 5-10. It must be emphasised that these histograms give no indication of the numbers of mitotic cells, and although no attempt was made to measure the mitotic index, it was clear that all treatments reduced the number of mitotic cells to a substantial degree. The results obtained with each inhibitor will now be described in turn.

**Camptothecin (Fig. 2)**

When applied to lymphocyte cultures at concentrations between 0.5 μM and 15 μM in the absence of colchicine, camptothecin reduces the number of mitotic cells to a very low level. Variations in the proportions of cells at different mitotic stages shown in the histogram are therefore unlikely to be of any significance. In the presence of colchicine, camptothecin appears to reduce the number of mitotic cells somewhat, but has no effect on the proportions of cells at the different stages of mitosis.

**Nalidixic acid (Fig. 3)**

Nalidixic acid, in the presence or absence of colchicine, causes a moderate reduction in the number of mitotic cells. In the absence of colchicine, the proportions at the different mitotic stages do not appear to be changed significantly. In the presence of colchicine, however, some remarkable changes take place. Metaphases with divergent chromatids, which comprise 37% of the control, are virtually eliminated by either 2.15 mM or 4.3 mM nalidixic acid. There is a substantial increase in the proportion of prophase cells, but most interestingly, most of the metaphase cells, which in the controls have parallel chromatids (Fig. 4b), have what are here termed fused chromatids after nalidixic acid treatment (Fig. 4a). Although the chromatids have the typical shape of metaphase chromosomes, there is no sign of a split between the chromatids. This appearance was only found as a very rare variant on control slides, or indeed after treatment with any other compound.
Fig. 1. Light micrographs defining the classification of the different stages of mitosis used in this study. (a) Early prophase: long tangled chromosomal threads occupying a circular, nucleus-shaped area; (b) late prophase: chromosomes still elongated, but individually recognisable, and occupying a more irregular area; (c) metaphase with fused chromatids; contracted chromosomes with no visible space between chromatids; (d) metaphase with parallel chromatids; (e) metaphase with divergent chromatids in some or all chromosomes; (f) anaphase: separation of centromeres of many or all chromosomes, with or without separation of the daughter groups of chromosomes; (g) telophase: complete separation and rounding up of daughter groups of chromosomes. Bars, 10 μm.
Fig. 2. Histograms showing the proportions of mitoses in prophase, early metaphase (with fused or parallel chromatids), and late meta-telophase (metaphases with divergent chromatids, anaphases, and telophases), after treatment of cultures with camptothecin at the concentrations indicated, in the presence (a) or absence (b) of colchicine.

Fig. 3. Histograms showing the proportions of mitoses at different stages after treatment of cultures with nalidixic acid, in the presence (a) or absence (b) of colchicine. For further explanation, see the legend to Fig. 2.

Fig. 4. Scanning electron micrographs of metaphase chromosomes: (a) from a culture treated with nalidixic acid, showing the lack of any splitting between the chromatids ("fused chromatids"); (b) from a control culture, grown without nalidixic acid, showing normal separation of the chromatids. Bars, 10 μm.
Chromosome segregation

Control 1nM 5j|1M 10|1M 20 nM
Ethidium concentration

Ellipticine (Fig. 6)
The results with ellipticine are generally similar to those obtained with ethidium, except that there is a substantial reduction in the number of mitotic cells as the ellipticine concentration is increased (40 and 100 μM ellipticine resulted in virtually complete suppression of mitosis).

Daunomycin (Fig. 7)
At concentrations between 4 and 100 nM, daunomycin has little effect on the number of mitotic cells, or on the proportions at different stages of mitosis. However, at concentrations of 0.4 μM and above, mitosis is suppressed almost completely.

Mitoxantrone (Fig. 8)
Treatment of cultures with mitoxantrone reduces the number of mitotic cells, and indeed largely suppresses mitosis in the absence of colchicine at the highest concentrations tested (1 and 2 μM). In the absence of colchicine, there is no significant alteration in the proportions of the different mitotic stages. In the presence of colchicine there is a suppression of prophase with increasing mitoxantrone concentration, and a slight increase in the proportion of metaphases with divergent chromatids.

Amsacrine (Fig. 9)
Amsacrine, at concentrations from 1-5 μM, almost completely suppresses mitosis in the absence of colchicine. The same concentrations of amsacrine, in the presence of colchicine, cause a small reduction in the number of mitoses, and completely suppress prophases. No substantial changes in the proportions of later stages were found.

Etoposide (Fig. 10)
Etoposide, at concentrations from 1-20 μM, almost
completely suppresses mitosis in the absence of colchicine. In the presence of colchicine, there was some reduction in the total number of mitoses, and at the highest concentration of etoposide (20 μM) complete elimination of prophases. The proportion of metaphases with divergent chromatids increased slightly.

**Inhibition of chromosome condensation**

Although the primary purpose of this study was to investigate the effect of topoisomerase II inhibitors on chromosome segregation, incidental observations were made on various other points. Most of the inhibitors...
tested appeared to inhibit chromosome condensation, reflected in an increase in the number of mitoses with long chromosomes showing a chromomeric structure. This was particularly clear with amsacrine and daunomycin (Fig. 11), but was not observed with camptothecin or etoposide, and the effect was small with nalidixic acid. In the case of ethidium, and perhaps also ellipticine, this effect is shown as an increase in the proportion of prophase cells.

Chromosome damage
Several inhibitors caused chromosome damage (Fig. 12). This was particularly noticeable with amsacrine and etoposide, but was also induced by mitoxantrone, daunomycin, and the topoisomerase I inhibitor camptothecin. No chromosome damage was noticed in the experiments with ethidium, ellipticine or nalidixic acid.

Discussion
The experiments described in this paper were designed to test the effects of topoisomerase inhibitors on mammalian chromosome segregation in two ways. Firstly, treatment of cultures with inhibitors in the absence of colchicine might be expected to lead to an accumulation of metaphase cells: the spindle might pull, but fail to separate, the chromosomes (cf. Uemura and Yanagida, 1986). Secondly, in the presence of colchicine, metaphases will accumulate in the absence of the spindle, which has been destroyed by the colchicine; however, under these conditions a characteristic divergence of the chromatids is seen, which is taken to be a manifestation of the segregation of the chromatids (but not of the centromere). The 5 h treatment with colchicine, and with inhibitors of topoisomerases, was chosen to maximise the effects just described. However, since the G2 phase of human lymphocyte cultures is in the region of 3 h (Kikuchi and Sandberg, 1964; Younkin, 1975), cells blocked in metaphase after 5 h treatment are likely to be derived mainly from cells in G2 at the start of treatment, with a proportion of S phase cells. Although it has been assumed that these cells form a uniform population in their response to inhibitors, there is no direct evidence for this, and indeed the procedure could conceivably select a population of mitotic cells that shows reduced susceptibility to topoisomerase inhibitors. Even if this were so, however, the principle behind the present experiments would not be invalidated, although higher concentrations of reagents might be needed to produce an observable effect. In practice, there is a limit to the extent to which concentrations of inhibitors can be increased, as the highest concentrations tested often caused almost complete elimination of mitotic cells (results not shown), and in many cases produced extensive chromosome damage before this limit was reached.

Four main effects of topoisomerase inhibitors on mitosis can be recognised in the present experiments (Table 2). The first is inhibition of entry into mitosis, seen as a loss of prophase, and a reduction in the total...
number of mitoses. This was a common, but not universal effect, which has already been described for many inhibitors (Grieder et al. 1977; Rowley and Kort, 1989; del Bino et al. 1991; Zucker and Elstein, 1991).

The second effect is inhibition of chromosome condensation. This effect has also been reported previously (Wright and Schatten, 1990; Charron and Hancock, 1990), and is expected on theoretical grounds, although measurements will be required to assess the degree of inhibition of condensation caused by the different compounds. It should be noted that the topoisomerase I inhibitor, camptothecin, does not appear to inhibit chromosome condensation. A third effect induced by several, but not all, the compounds tested is the induction of chromosome damage; again there have been previous reports of this phenomenon (Huang et al. 1973; Kao-Shan et al. 1984; Pommier et al. 1988; Charron and Hancock, 1991).

The main effect sought in this study, the inhibition of chromosome segregation, has been the most difficult to establish. Evidence that it is occurring comes from a reduction in the proportion of metaphases with divergent chromatids, and a reduction in the proportion of anaphases. These effects are, however, only seen with certain compounds. Most interesting, however, is the observation of metaphase chromosomes with fused chromatids after treatment with nalidixic acid. All these observations suggest that chromosome segregation might be inhibited. On the other hand, there is no evidence that any of the topoisomerase II inhibitors tested caused an accumulation of cells in metaphase, and in fact most caused some reduction, often very

Fig. 12. Metaphases showing damaged chromosomes after treatment with (a) mitoxantrone, (b) amsacrine. Bars, 10 μm.

Table 2. Summary of effects of topoisomerase inhibitors on mitotic chromosomes

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Camptothecin</th>
<th>Nalidixic acid</th>
<th>Ethidium bromide</th>
<th>Ellipticine</th>
<th>Daunomycin</th>
<th>Mitoxantrone</th>
<th>Amsacrine</th>
<th>Etoposide</th>
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<tbody>
<tr>
<td>Inhibition of entry into mitosis</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Loss of prophase</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Reduction in number of mitoses</td>
<td>+</td>
<td>-</td>
<td>(+)</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Increase in prophase</td>
<td>-</td>
<td>+</td>
<td>(+)</td>
<td>(+)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Elongated chromatids</td>
<td>-</td>
<td>( + )</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Chromosome breakage and rearrangement</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Inhibition of segregation</td>
<td>-</td>
<td>+</td>
<td>(+)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Fused chromatids</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<td>-</td>
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<tr>
<td>Accumulation of metaphases</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Reduction in metaphases with divergent chromatids</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Reduction of anaphases</td>
<td>( + )</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>( + )</td>
<td></td>
</tr>
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</table>

-, No discernable effect; (+), possible slight effect; +, moderate effect; ++, strong effect.
substantial, in the number of mitoses. This is most easily interpreted as a block in G2, as already described by several authors (Grieder et al. 1977; Rowley and Kort, 1989; del Bino et al. 1991; Zucker and Elstein, 1991), but with little or no effect on cells already in mitosis, which apparently pass through mitosis more or less normally. The results described in this paper do not, therefore, support the hypothesis that topoisomerase II is involved directly in the segregation of mammalian chromosomes at mitosis. This is in agreement with the results of Rowley and Kort (1989), who, however, used somewhat non-specific reagents. Wright and Schatten (1990) also failed to find an effect of teniposide on mitotic segregation, although this compound inhibited meiotic segregation, in the clam Spisula.

The assay used in this study for assessing inhibition of segregation is similar to that described by Miller and Adler (1989), although the method used here was devised independently. Miller and Adler used their method to study spindle poisons, and it might be argued that, in the present study, when microtubules were not destroyed by the use of colchicine, the spindle could still function to pull the daughter chromosomes apart at anaphase, breaking any DNA connections between them mechanically. Such a process would be expected to lead to damaged chromosome fragments, which might be visible at anaphase, and appear as micronuclei at the next interphase. The latter point has not been studied yet, but no evidence was found in any of the experiments described here for faulty segregation at anaphase. However, Wilson et al. (1984) reported the induction of micronuclei by several topoisomerase II inhibitors.

Downes et al. (1991) have recently reported that inhibitors of topoisomerase II do, in fact, interfere with chromosome segregation in mammalian cells, and that high concentrations of etoposide or amsacrine prevent anaphase separation of chromatids. There are two possible explanations of the difference between the results of Downes et al. and those reported in this paper. Firstly, they used much higher concentrations of topoisomerase II inhibitors (20-60 µM etoposide, instead of 1-20 µM used here, and 40 µM amsacrine, instead of 1-5 µM used here), although treatment was for a much shorter time. It might simply be that the concentrations used in my experiments were inadequate to produce an effect on segregation. However, the concentrations used in the present experiments were comparable with those reported in the literature, and it was not practicable to use higher concentrations as these completely eliminated mitotic cells. On the other hand, even 1 µM amsacrine or etoposide induce substantial chromosome damage if used before mitosis.

In fact, Silvestrini et al. (1970) showed that treatment of cultures with high concentrations of Adriamycin caused the accumulation of metaphases showing extensive chromosomal damage, while Charron and Hancock (1991) have reported defective segregation after treatment with teniposide, due to the inability of rearranged chromosomes to segregate normally. Indeed, Downes et al. (1991) found a similar phenomenon themselves using a low concentration of etoposide. However, Downes et al. (1991) in their critical experiments applied the topoisomerase inhibitors only during mitosis, thus eliminating the problem of chromosome damage. They point out, nevertheless, that effects on segregation are only perceptible at concentrations of etoposide that are lethal after a period comparable with the duration of mitosis, and the possibility must be borne in mind that their results could be affected by the unhealthy condition of the cells rather than being entirely due to inhibition of topoisomerase II.

In the present experiments, the effects of several topoisomerase II inhibitors have been compared. It was felt that, since most of them have side-effects other than inhibition of topoisomerase II, it would be necessary to look for effects common to several compounds to be certain that an effect on topoisomerase II was being observed. In addition, different compounds act on topoisomerase II in different ways. For example, Adriamycin, a compound closely related to daunomycin, also inhibits cytochrome c oxidase in mitochondria (Goormaghtigh et al. 1982). Mitoxantrone appears to inhibit mainly the 180 kDa form of human topoisomerase II, which does not vary significantly in amount during the cell cycle, but is largely without effect on the 170 kDa form (Harker et al. 1991). The latter does vary in amount during the cell cycle, and is therefore more likely to be the form responsible for mitotic events. In addition mitoxantrone inhibits polymerisation of microtubules (Ho et al. 1991), and therefore might be expected to cause metaphase arrest independently of any action on topoisomerase II. Nalidixic acid, as well as having some inhibitory effect on topoisomerase II, is in fact primarily an ATPase inhibitor (Gallagher et al. 1986). Since it is the only compound tested that promotes chromatid fusion (strictly speaking, the inhibition of chromatid separation) this phenomenon, although potentially interesting in its own right, is unlikely to be the result of inhibition of topoisomerase II. Even compounds such as etoposide and VM-26 (teniposide), which are regarded as being among the most specific topoisomerase II inhibitors, also inhibit phosphorylation of histones, a process associated with chromosome condensation, although it is not clear whether this is a direct effect (Lock and Ross, 1990; Roberge et al. 1990). Amsacrine, another topoisomerase II inhibitor regarded as being highly specific, also inhibits aldehyde oxidase (Gormley et al. 1983). It seems, therefore, that it is not possible to rely on one single topoisomerase II inhibitor to investigate the importance of topoisomerase II in cellular processes.

In conclusion, a variety of compounds reported to inhibit topoisomerase II induce four effects on human mitotic cells: inhibition of entry into mitosis; chromosome breakage and rearrangement; inhibition of chromosome condensation; and inhibition of chromosome segregation. None of the compounds tested produced all these effects; this may have been due to differences in their actions on topoisomerase II, or the use of inappropriate concentrations of the compounds.
There is also the possibility that some inhibitors were also acting on substrates other than topoisomerase II. Evidence that the inhibitors tested were inhibiting segregation was least satisfactory, since although there was some evidence that segregation was inhibited in cells treated with colchicine, it appeared that in the absence of colchicine cells could pass through mitosis without difficulty. In this context it is worth noting that Koshland and Hartwell (1987) found that the daughter DNA molecules in a yeast minichromosome were decatenated before anaphase, possibly shortly after S phase. If this finding could be extrapolated to mammalian chromosomes, there would be no reason to expect that topoisomerase II inhibitors would have any effect on the anaphase separation of chromosomes. The proposal by Sumner (1991) that topoisomerase II acts on centromeric satellite DNA at the start of anaphase to produce chromosome segregation would not, therefore, be supported; instead it would seem more likely that initiation of segregation is due to the action of a "chromatid separase" (Ostergren and Andersson, 1973) on the proteins of centromeric heterochromatin. The results reported here do not, therefore, agree completely with those described for yeasts (di Nardo et al. 1984; Uemura and Yanagida, 1986; Uemura et al. 1987; Holm et al. 1989) and more recently for mammalian cells (Downes et al. 1991), in which segregation appears to be defective if topoisomerase II is inhibited. It should be noted, however, that the studies just cited did not investigate individual chromosomes, and the possibility that abnormal segregation might be a consequence of the induction of chromosome damage must be borne in mind.

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