Ann. Ist. Super. Sanità
Vol. 25, N. 1 (1989), pp. 41-50

BIOCHEMICAL AND ENZYMATIC ASPECTS OF CAFFEINE AND CAFFEINE DERIVATIVES INDUCED DNA STRAND BREAKS

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Summary. - *The results presented here point to the difficulties that exist in connection with the identification of the molecular target of caffeine. Our data support the evidence that caffeine and caffeine derivatives cause DNA-protein cross-links (DPC) in whole mammalian cells or in isolated nuclei. These DPC have the same properties (saturability, reversibility and temperature-dependence) as those produced by an enzymatic inhibition. The experiments performed in reconstituted systems, in the presence of purified DNA topoisomerase II, do not support the original hypothesis that this enzyme might be a possible target for this class of drugs. We suggest the possibility that other DNA metabolism enzymes are involved in the biological effects of caffeine and caffeine derivatives. Further biochemical and molecular data are necessary to identify which of these enzymes is in fact affected.*

Riassunto (Aspetti biochimici ed enzimatici delle rotture a singolo filamento del DNA indotte dalla caffeina e suoi derivati). - *I risultati presentati in questo lavoro puntualizzano le difficoltà esistenti nell'identificare, a livello molecolare, il bersaglio della caffeina. I nostri dati mostrano che caffeina e derivati causano "cross-links" DNA-proteine (DPC) in cellule di mammifero o in nuclei isolati. Questi DPC hanno le stesse proprietà (saturabilità, reversibilità e temperatura-dipendenza) dei DPC prodotti da inibizione enzimatica. Gli esperimenti condotti in "sistemi ricostituiti" in presenza di topoisomerasi II purificata, non concordano con l'ipotesi originale che tale enzima possa essere il bersaglio di questa classe di composti. Noi suggeriamo la possibilità che altri enzimi legati al metabolismo del DNA siano coinvolti negli effetti biologici di caffeina e caffeina derivati. Ulteriori dati biochimici e molecolari sono necessari per identificare l'enzima bersaglio.*

Introduction

In spite of extensive investigations in several biological systems, the molecular basis of the multiple effects of caffeine and its derivatives on DNA and chromosomes remains elusive. It has been demonstrated [1-3] that caffeine and many other methylated oxypurines (MOPs) produce chromosomal aberrations in plant and animal cells. The effect is independent of DNA and chromosome replication (G_2 and prophase represent the most sensitive stages), but it is temperature-dependent, the highest frequency of aberrations being obtained at 22-27 °C in mammalian cells. The effect is also further strongly dependent on the ATP level of the cell [1-3].

Furthermore, post-treatment with caffeine in mammalian cells (cultured human lymphocytes) during G_2 strongly enhances the frequencies of aberrations induced both by X-rays and by alkylating agents given at earlier stages of the cell cycle. In contrast, post-treatment with caffeine during S-phase produces little or no enhancement in these cells [1, 4]. A clear S-phase effect is found in plant cells [1-4].

It has been proposed that the S-phase effect may be ascribed to an inhibitory effect of caffeine on the gap-filling process of post-replication repair [5]. This effect may be important in allowing cells to escape the toxic effects of DNA-damaging agents or in permitting caffeine to reverse the inhibiting effects of chemical and physical mutagens on DNA synthesis [6, 7]. The potentiating effect of caffeine post-treatment during G_2 has been connected with the well-known ability of caffeine to reverse the G_2-delay induced by X-rays and chemical mutagens [8-10]. As a consequence of this effect a short time would be available for DNA repair and cells would arrive at mitosis with more unrepaired DNA-damage visualized as a higher frequency of aberrations [11].

In conclusion, although the effect of caffeine on DNA is probably one of the most thoroughly studied subjects [1-3] its complicated effects make it difficult to clarify its real mechanism of action.

Recently Kihlman and Andersson [3], inspired by some similarities, at the chromosomal level, between the effects of this class of methylated oxypurines and some antineoplastic drugs, have suggested that at least some of the effects of caffeine could be due to an interference with DNA topoisomerase II. DNA topoisomerase II are ubiquitous enzymes that control cellular

DNA topology. Each of these reactions involves the formation of enzyme-DNA complexes, in which topoisomerase II cleaves one DNA double-strand while binding covalently to the 5'-DNA termini and allowing the passage of an intact DNA double-strand through the DNA break made by the enzyme [12-14]. It is believed that DNA intercalating agents currently used in cancer chemotherapy and demethylepipodophyllotoxins (VP16 and VM26) trap the covalent intermediates of the above reaction and that these intermediates are detected as protein-associated DNA strand breaks (DPC) in mammalian cells [15-21] which are equivalent to the enzyme-DNA complexes observed in purified systems [16]. In addition, topoisomerase II inhibitors produce sister chromatid exchanges (SCE), chromosomal aberration, increased chromosome number, and cell killing; [22, 23] whether all these effects are consequences of topoisomerase II inhibition is not yet clearly known. However, accumulating evidences suggest that the formation of drug-induced SCEs and chromosomal aberrations are related to topoisomerase II inhibition [22]. Therefore, it is tempting to assume that topoisomerase II inhibition leads to both drug-induced protein associated DNA strand-breaks and chromosomal abnormalities. Whether the chromosomal rearrangements are due directly to the topoisomerase II-mediated DNA breaks by an enzyme subunit exchange mechanism [23] or to some indirect mechanism remains to be demonstrated.

This paper reviews work from our laboratories providing evidence to test the hypothesis that MOPs could interfere with DNA topoisomerase II. Our initial work was in the murine L1210 leukemia system. Subsequently, we have performed experiments in quiescent and mitogen stimulated T human lymphocytes. Our most recent efforts have examined the molecular interactions between DNA, topoisomerase II and MOPs in reconstituted systems *in vitro*. Using purified mouse leukemia L1210 topoisomerase II and SV40 DNA we measured both the covalent DNA binding of topoisomerase II in a filter binding assay and looked at the location of the trapped topoisomerase II-DNA complexes using [^{32}P]-end labeled SV40 DNA and DNA agarose electrophoresis. We have also measured the effect of MOPs on the decatenation activity of calf thymus DNA topoisomerase II. Intercalative binding of MOPs to DNA was investigated by use of a DNA unwinding assay.

Protein-associated DNA cleavage in murine L1210 leukemia cell nuclei

The initial observations by Ross *et al.* [24, 25] were critical in establishing that a novel form of DNA cleavage was produced by DNA intercalating agents.

Intercalating agents are drugs that interdigitate between adjacent DNA base pairs and untwist the DNA helix. They are among the most potent clinically active antineoplastic agents. Adriamycin, daunomycin, ellipticine and mAMSA [4'(9-acridinylamino)methanesulfonm-anisidide] are some of these agents. When Ross *et al.* [24, 25] tested the effects of adriamycin on mouse leukemia L1210 cells, they noted, upon quantitation of the frequencies of single-strand breaks (SSB) and DPC, that the two lesions were present in approximately equal amounts. The only way to account for these results was to assume that the strand-breaks and the DNA-protein cross-links were associated in some way, so that there was one linked protein for every pair of successive single strand-breaks [25]. Furthermore, these unique protein-associated DNA breaks were common to a whole series of intercalating compounds but not to drugs bound to DNA without intercalation or that inhibited DNA synthesis without binding to DNA [25]. Once Ross *et al.* [25] had obtained evidence for both a spatial and quantitative relation between the strand-breaks and cross-links as revealed by the alkaline elution technique, developed by Kohn *et al.* [26], the authors again suggested that the protein may represent a topoisomerase like enzyme which was responding to the topological distortions imposed by the intercalation of the drug into DNA. The nonintercalating antitumor epipodophyllotoxins (e.g. etoposide (VP16) and teniposide (VM26)) were also subsequently shown to generate strand-breakage and DNA protein cross-linking analogous to that described for the other drugs [27].

A number of characteristics which might be predictive of a topoisomerase-mediated mechanism have been demonstrated in studies employing cultured cell lines. Drug-induced production of the DPC is, independently of drug uptake, a temperature-dependent phenomenon and one that becomes saturated with increased drug concentration [28]. When the drug effects were examined in L1210 isolated nuclei, they were found to depend on the presence of magnesium and were stimulated by ATP [29, 30].

Ellipticine and mAMSA were found to induce equivalent frequencies of SSB, DSB and DPC. These lesions were spontaneously reversible upon drug removal, and the reversal did not require the presence of ATP [30]. In addition ellipticine determined a biphasic effect; at low concentration it stimulated the formation of protein-linked strand-breaks but at high concentration it inhibited and reversed the production of protein-linked strand-breaks caused by the drug itself and by other intercalating drugs, such as mAMSA [30].

The first step of our study was to see whether MOPs acted on L1210 cell nuclei in the same ways described above for intercalating agents. Particularly we referred our results to those obtained from work with ellipticine, a well-known inhibitor of topoisomerase II.

For this set of experiments we selected three MOPs, namely caffeine, 8-methoxycaffeine (8-MOC) and 8-chlorocaffeine (8-CC). We measured the formation of DSB since they are the most satisfactory measure of

altered topoisomerase II action and they are less susceptible than SSB to spurious nicking of DNA during incubation. Moreover, they could be measured over a wider range of drug concentrations by a single filter elution protocol [26]. Isolated L1210 cell nuclei were used because the DSB frequencies produced by intercalators are usually similar to those found in whole cells [30] and because the nucleus preparation avoids the problems of drug cellular uptake or metabolism [30]. In the course of this study, we observed that caffeine, 8-MOC and 8-CC were able to produce DSB at concentrations above 50 μM; DSB increased with increasing drug concentration; at higher concentrations DSB decreased and reached the background level at a concentration of 3 x 10^{-2} M (Fig. 1). A similar bellshaped curve has been observed in the same system for ellipticine [30].

Given our hypothesis that interaction of MOPs with topoisomerase II was necessary for the production of DNA-breakage, we considered it important to define the intranuclear conditions that might influence enzymatic activity. We have found drug activity to be dependent on magnesium and to be stimulated by low concentrations of ATP (0.5 mM). This effect is temperature-dependent and spontaneously reversible upon drug removal (Table 1). Finally the three compounds were able to induce SSB, when a proteolitic step was included during the elution assay (Table 1).

All the properties observed for caffeine, 8-MOC and 8-CC were very similar to those observed for ellipticine [30].

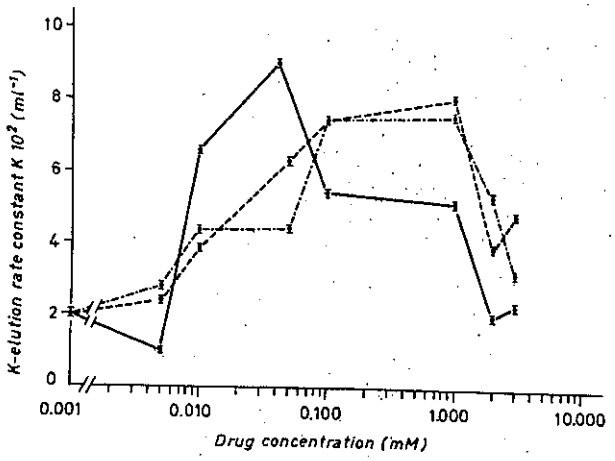

Fig. 1. - DNA double-strand breaks (DSB) produced by caffeine (—·—), 8-MOC (———), 8-CC (— —) in isolated L1210 cell nuclei. Nuclei were treated with MOPs for 30 min at 22 °C. Error bars denote standard errors of at least three independent determinations. All results are espressed as elution rate K values (mean SE). K is given by the following formula:

$$K = \frac{-\ln(\text{fraction of DNA retained on filter})}{V}$$

where K is the average elution rate constant (ml^{-1}) of DNA and V is the elution volume in ml. The above formula reflects the assumption of a first order kinetics for DNA elution, as a first approximation [26].

Table 1. - *Elution rate K induced by caffeine, 8-methoxycaffeine (8-MOC), 8-chlorocaffeine (8-CC) or ellepticine in isolated L1210 nuclei in different experimental conditions*

	Caffeine (1 mM)	8-MOC (0.05 mM)	8-CC (1 mM)	Ellipticine (0.01 mM)	Nuclear buffer
Standard condition *(a)*	7.30 ± 0.53	9.02 ± 0.08	8.15 ± 0.04	14.38 ± 0.88	1.98 ± 0.28
Minus Mg^{++} *(b)*	2.10 ± 0.12	2.34 ± 0.33	2.82 ± 0.88	2.20 ± 0.46	2.05 ± 0.38
Plus ATP 0.5 mM *(c)*	10.96 ± 1.15	10.73 ± 1.64	12.03 ± 1.59	18.73 ± 3.41	1.57 ± 0.32
Incubation at 4 °C *(d)*	1.14 ± 0.36	2.84 ± 0.40	0.80 ± 0.15	5.22 ± 0.39	1.9 ± 0.17
(e)	3.31 ± 0.57	2.22 ± 0.34	3.75 ± 0.49	2.71 ± 0.36	2.76 ± 0.66
pH = 12.3 *(f)*	9.08 ± 0.35	9.25 ± 1.06	9.88 ± 1.54	13.17 ± 0.84	1.98 ± 0.14
Minus proteinase K; pH = 12.3 *(g)*	2.88 ± 0.45	2.12 ± 0.29	3.16 ± 0.22	2.56 ± 0.37	2.95 ± 0.57

(a) Nuclei were incubated for 30 min at 22 °C in complete nuclear buffer 150 mM NaCl, 1 mM KH_2PO_4, 1 mM EGTA, 0.1 mM DTT (pH = 6.4). The lysing solution contained proteinase K 0.5 mg/ml (for 30 min of incubation). Elution was performed at pH 9.6.
(b) As in *(a)*, but without Mg^{++} in the nuclear buffer. In this case nuclear buffer contained 0.5 mM spermidine to protect nuclei from endogenous DNA breaks in the absence of Mg^{++}.
(c) As in *(a)*, but nuclei were preincubated at 22 °C for 5 min with ATP 1.0 mM. The incubation continued for 30 additional min with ATP 0.5 mM plus drug under testing.
(d) As in *(a)*, but nuclei were incubated at 4 °C.
(e) As in *(a)*, but after 4 washes of nuclei with nuclear buffer to remove the tested drug.
(f) As in *(a)*, but the elution was performed at pH 12.3.
(g) As in *(f)*, but proteinase K was omitted in the lysing solution.
Each experiment refers at least four independent determinations. All results are espressed as elution rate K values (media ± SE).

In addition at self-inhibitory concentrations MOPs were able to inhibit the DSB produced by ellipticine (Fig. 2).

Since, taken together, these results strongly indicate that the two classes of compounds (MOPs and ellipticine) present the same properties and might interact with the same class of nuclear targets, we worked to demonstrate that MOPs induce DNA-protein cross-links and that the strand-breaks and DNA-protein cross-links are present in approximately equal quantities as reported for all topoisomerase II inhibitors. For these experiments we have chosen 8-MOC because it is the most efficient methylated oxypurine in generating both DSB and SSB. The assay of DPC is based on the efficient adsorption of proteins at pH 12 to hydrofobic filters under appropriate conditions [31]. Nuclei were treated at 22 °C for 30 min with different concentrations of 8-MOC. They were subsequently exposed at 4 °C to 3000 rads X-rays which introduces an appropriate frequency of random SSB (Fig. 3). We found that 8-MOC increased the magnitude of the slowly eluting component. The interpretation of this effect is that 8-MOC caused DNA-protein cross-links. But when we looked at the concentrations giving SSB (Fig. 4) and DPC (Fig. 3) we observed that 8-MOC was more efficient in determining DPC than in causing SSB, in contrast to ellipticine which causes SSB and DPC at the same ratio. This is the first discrepancy between MOPs and antitumor topoisomerase II inhibitors. But it is no small discrepancy because the DNA intercalators, ellipticine, and mAMSA induce equivalent frequencies of SSB and DPC in isolated L1210 cell nuclei. This experimental evidence reported for 8-MOC, would be contrary to an involvement of DNA topoisomerase II in the formation of the protein-linked breaks caused by MOPs.

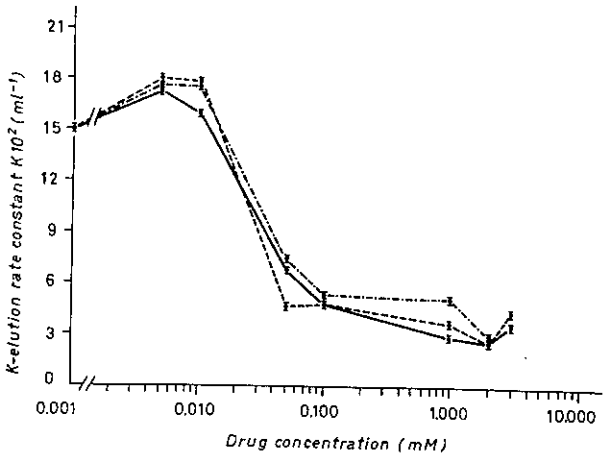

Fig. 2. - Inhibition of ellipticine-induced DSB production by increasing concentrations of caffeine, 8-MOC, 8-CC. Isolated nuclei were treated for 30 min at 22 °C with various concentrations of caffeine (— —), 8-MOC (———), 8-CC (—·—) together with 10 μM ellipticine.

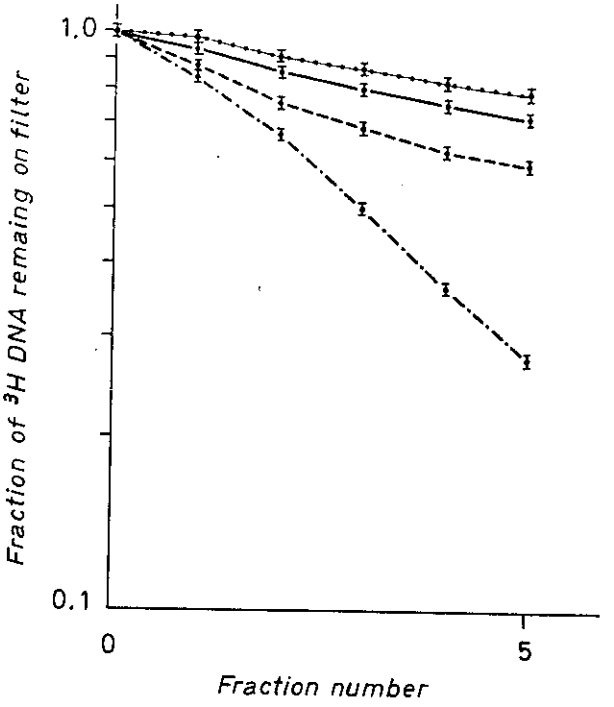

Fig. 3. - Effect of 8-MOC treatment on the residual retention of DNA from L1210 nuclei irradiated with 3000 rads. Following treatment (for 30 min at 22 °C) the nuclei were chilled, rinsed, irradiated with 3000 rads and eluted from PVC filters. Concentrations of 8-MOC were the same as shown in Fig. 2.
0-0 controls; 10 μM 8-MOC + 3000 rads (— —); 50 μM 8-MOC + 3000 rads (———); 3000 rads (—·—).

Biological effects

Relation to cell killing

Although the biochemical effects of topoisomerase II inhibitors have become better understood in recent years, an unquestionable causal nexus between drug-induced protein linked strand breaks and cytotoxicity has yet to be established. A number of experimental approaches have been used to establish a quantitative relationship between drug-induced topoisomerase II inhibition and cytotoxicity [32]. Some of these observations are consistent with a causal relationship between topoisomerase II-mediated damage to DNA and the cytotoxicity of antitumor DNA intercalators and 4'demethylepipodophyllotoxins [33-35]. However, some other studies [36, 37] have suggested that drug-induced DNA SSB were not necessarily equally lethal among the various classes of topoisomerase II inhibitors and in different cell lines. Indeed Pommier *et al.* [38] have demonstrated a good correlation between DNA-DSB and cytotoxicity for mAMSA and 5 iminodaunorobucin, but Ross *et al.* [39] have found that ellipticine produces a great frequency of strand breaks but it is only weakly cytotoxic. According to our initial project, the discovery of the molecular target of caffeine and derivatives, we carried out a study seeking to compare relative effects on DNA (induction of DSB) with relative effects on cell killing. We

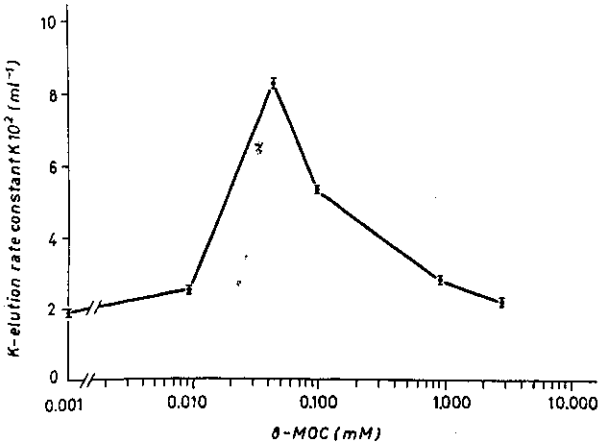

Fig. 4. - DNA single-strand breaks (SSB) produced by 8-MOC. Nuclei were treated with 8-MOC for 30 min at 22 °C. Error bars denote standard errors of at least three independent determinations.

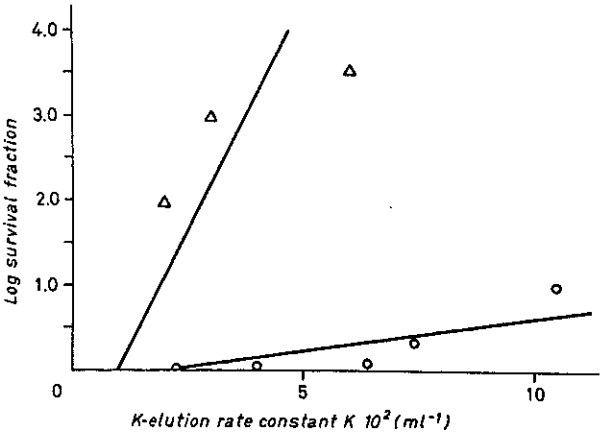

Fig. 5. - Relationships between cytotoxicity and DNA double-strand breaks for 8-chlorocaffeine and adryamicin in L1210 cells. Cloning efficiency after exposure of cells for 1 h to various concentrations of 8-CC and adriamycin was plotted against the elution rate constant K (o) 8-CC; (▲) adriamycin.

examined a large number of MOPs (e.g. caffeine, 8-MOC, 8-CC, 8-ethoxycaffeine, 8-methylthiocaffeine, teophylline and teobromine) and compared their effects to that caused by adriamycin. The cell-killing effects of the various drugs were examined by soft agar colony formation assay. MOPs are not cytotoxic up to a concentration of 3-5 mM, but are able to induce DNA-DSB in a concentration-dependent manner in L1210 cells already at lower concentrations. To examine the correlation between DNA damage and cytotoxicity, cell killing was plotted as a function of DSB frequency. For this we chose only 1 MOP namely 8-CC; adriamycin was used as a reference standard. As shown in Fig. 5 the results did not demonstrate a correlation between DSB formation and cytotoxicity. This happened only for adriamycin. These results seem to suggest a different relationship, between DNA double-strand breaks and cytotoxicity, from the one observed for antitumor topoisomerase II inhibitors [32, 38]. On the other hand, the effects induced by MOPs seem to resemble those obtained with trans-platinum [31]. The trans-Pt [31] complex is in fact an excellent mean of producing high frequencies of DNA-proteins cross-links but it is only weakly cytotoxic.

Role of proliferation in determining sensitivity to caffeine and caffeine derivatives

As previously reported in the "Introduction", caffeine and many MOPs are capable of producing chromosomal aberrations in plant and animal cells, independently of DNA and chromosome replication, G_2 phase and prophase being by far the most sensitive stages.

Several authors have suggested a possible correlation between the activity of mammalian topoisomerase II and cell proliferation. Duguet *et al.* [40] showed that the low level of enzyme activity in liver increased significantly, after partial hepatectomy. Taudou *et al.* [41] found a low level of enzyme activity in G_2 guinea pig lymphocytes that increased dramatically after mitogenic stimulation. Finally Sullivan *et al.* [42] and Markovits *et al.* [43] demonstrated a proliferation-dependence of VP16 and mAMSA-induced protein linked DNA breaks in CHO cells and in 3T3 cell. More direct proof of this relationship, however, has come from a series of elegant studies by Heck and Earnshow [44] employing an antibody probe to measure the levels of topoisomerase II in several transformed and developmentally regulated normal cell types. They demonstrated that, when peripheral blood lymphocytes (which lack detectable topoisomerase II) commence proliferation, they express topoisomerase II *de novo* and its level rises coordinately with the onset of DNA replication. This study clearly showed that topoisomerase II is a sensitive and specific marker for proliferating cells. Inspired by these findings we undertook a study in which we correlated drug sensitivity in terms of cleavable-complex formation, with cell proliferation. Cleavable complex formation in whole cells was evaluated as DNA-DSB frequency using the alkaline elution technique, as above described. As a proliferating system we chose the human lymphocytes. It is well-known that peripheral blood lymphocytes (in G_0 phase) may be stimulated to enter the cell cycle in response to an immunological challenge *in vivo*, or in response to a specific mitogen *in vitro*. T-human peripheral blood lymphocytes were obtained from normal human volunters, purified by centrifugation over Ficoll hypaque gradient of E-rosetting cells and finally stimulated with 1% vol/vol PHA. We observed the formation of DNA-DSB induced by 8-MOC only 72 h after the addition of PHA when 80% of T-human lymphocytes were in G_2 phase. On the contrary the etoposide was already clearly active at 48 h (S phase) (Table 2).

Table 2. - *DNA double-strand break frequency in purified human T-lymphocytes induced by VP16 and 8-MOC as a function of cell proliferation*

Treatment		Quiescent (Time 0 h)	(Time 14 h)	Proliferative (Time 48 h)	(Time 72 h)
Untreated		3.25 ± 0.47	3.80 ± 0.62	3.28 ± 0.14	1.67 ± 0.73
VP16	40 μM	4.94 ± 0.67	6.98 ± 0.58	9.24 ± 0.73	9.07 ± 1.61
8-MOC	10 μM	3.26 ± 0.56	-	-	4.50 ± 0.65
	50 μM	3.32 ± 0.86	0.95 ± 0.13	2.16 ± 0.24	8.55 ± 1.12

Cells were either quiescent (time 0) or proliferating (time 14 or 48 or 72 h), following addition of PHA. Cells were exposed to VP16 or 8-MOC for 60 min at 37 °C. DNA cleavage was quantified using neutral elution technique and was expressed as elution rate constant K.

The findings seem to suggest that 8-MOC is active a little later in the cell cycle than topoisomerase II inhibitors.

Reconstituted systems

Since the above experiments, which were generally performed in cellular and subcellular systems (nuclei and cells), gave conflicting results and did not clarify the nature of the molecular target of caffeine, we investigated the effects of caffeine and derivatives upon purified topoisomerase II.

We measured the cleavable complex formation by determining the binding of topoisomerase II to [^{3}H]-labeled SV40 DNA in a filter binding assay [45] derived from that of Minford *et al.* [16]. It utilizes the property of poly(vinylchloride) filters to retain protein-bound DNA in the presence of 2% sarkosyl and 2 M NaCl, without retaining free DNA [26] cleavable complex formation was quantified by determining this covalent binding. In contrast to mAMSA, our positive control, 8-MOC did not stimulate, or slightly stimulated the formation of presumed covalent complexes between SV40 DNA and L1210 purified DNA topoisomerase II (Fig. 6) [45].

This was the first direct evidence against our original hypothesis that topoisomerase II might be a possible target for this class of drugs.

We continued our study by looking at the location of the trapped topoisomerase II-DNA complexes using [^{32}P]-end-labeled SV40 DNA and DNA agarose electrophoresis [46]. The drug induced-DNA cleavage complexes of topoisomerase II tend to occur at preferential sites along the DNA sequence [47]. The sites of double-strand cleavage can be located by means of agarose gel electrophoresis of a [^{32}P]-end-labeled linear DNA fragment. Analyses of those gels have shown that the preferred sites of cleavage along the DNA sequence depend on the chemical class of the inhibitory drug, on the concentrations of the components of the system and on the presence of ATP [47].

The preferred sites of drug-stimulated cleavage are in most cases identifiable with cleavage sites produced by the enzyme without the addition of drugs. In the absence of drugs, however, the cleavage is usually at a very low level. Different sites are enhanced by different classes of drugs. For example mAMSA produces cleavage-site preferences which differ from those stimulated by ellipticine, or adriamycin or VP16 [46, 48, 49]. In our study caffeine, 8-MOC and 8-CC gave little or no stimulation for cleavage. We also obtained similar negative results working in the same system, but in the presence of 350 mM NaCl L1210 nuclear extract instead of purified topoisomerase II. Caffeine, 8-MOC and 8-CC were again devoid of any direct strand-breaking activity on SV40 DNA (Figs 7 and 8).

Taken together these results strongly suggest that the protein involved in the formation of DPC, induced by MOPs, is not DNA topoisomerase II.

DNA strand-passing

Eukaryotic tipe II DNA topoisomerase catalyzes decatenation of DNA circles in an ATP-dependent fashion. This assay used a mitochondrial DNA, kinetoplast DNA (KDNA) of *Chritidia fasciculata*, a trypanosomatid insect. This DNA exists in the form of networks of up to about 5000 interlocked covalently closed minicircles which are 2.5 kb long [30]. In this reaction topoisomerase II acts by passing one DNA segment through a double-strand break of another segment of the same or of a separate molecule. Since the antitumor intercalating agents a 4'-demethylepipodophillotoxins are able to inhibit this activity [16], we studied the ability of 8-MOC to interfere with the catalytic activity of purified calf thymus DNA topoisomerase II. In this experiments we observed that 8-MOC partially affected the strand-passing activity of topoisomerase II in KDNA decatenation assay (Fig. 9), although this may require a far higher concentration than does the induction of breaks in alkaline elution (see experiments performed with alkaline elution assay). Ellipticine and derivatives inhibit the decatenation catalyzed by topoisomerase II at concentrations around 2 μM (same range as active concentrations in alkaline elution) as opposed to 500 to 1000 M for 8-MOC. This is another discrepancy between the

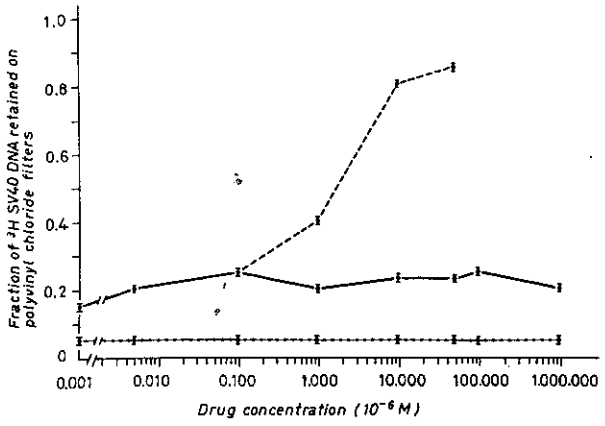

Fig. 6. - Effect of 8-MOC or mAMSA on topoisomerase II binding to SV40 DNA. Reaction mixtures (100 µl) contained 260 ng of topoisomerase II, 40 ng of ^{3}H-labeled SV40 DNA, and various concentrations of 8-MOC or mAMSA (positive control); incubation was for 30 min at 37 °C. Reaction were stopped by 20-fold dilution in 20 mM EDTA (pH 10) at 4 °C. DNA covalent binding was assayed as the fraction retained after a 3 ml wash of the filter with 2 M NaCl, 0.2% sarkosyl, and 0.02 EDTA, pH 10 (LS 10 fraction).

$$\text{covalent binding} = \frac{[\text{filter}]}{[\text{filter}] + [\text{LS10 fraction}] + [\text{EDTA fraction}]}$$

(——●——) DNA + different concentrations of 8-MOC; (———) DNA + topoisomerase II + different concentrations of 8-MOC; (— — —) DNA + topoisomerase II + different concentrations of mAMSA. Error bars represent the standard errors of at least three independent experiments.

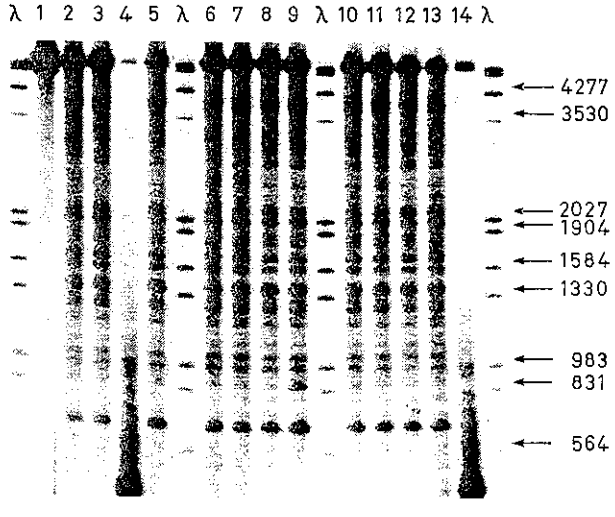

Fig. 7. - DNA double-strand break pattern of L1210 topoisomerase II in the absence or presence of methylated oxypurines in [^{32}P]-end-labeled SV40 DNA. SV40 DNA (lane 1) was reacted with purified L1210 topoisomerase II in the absence (lane 2) or presence of various concentrations of either caffeine (30, 300 µM in lanes 3 and 5), 8-MOC (10, 30, 100, 300 µM in lanes 6, 7, 8, 9), 8-CC (10, 30, 100, 300 µM in lanes 10, 11, 12 and 13) or mAMSA (10 µM in lanes 4 and 14). Reactions were performed in the presence of 1 mM ATP for 30 min at 37 °C and then stopped by adding SDS and proteinase K (1% and 0.5 mg/ml final concentrations, respectively). Reaction mixtures were further incubated for 30 min at 37 °C and run into a 1% agarose gel in tris-borate-EDTA buffer. The gel was then dried and autoradiographed: *HindIII/EcoRI* digest of lambda DNA. The size (in base pairs) of the fragments is indicated at the right of the picture.

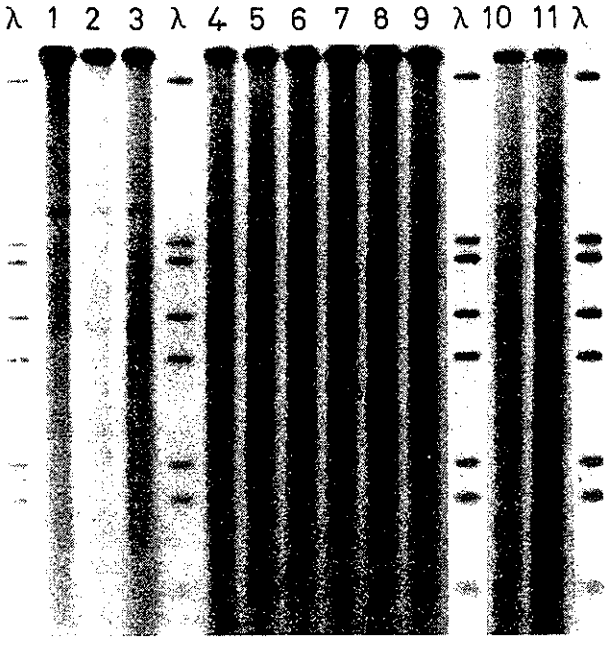

Fig. 8. - DNA double-strand breaks pattern of 350 mM NaCl L1210 nuclear extract in the absence or presence of 8-MOC in [^{32}P]-end-labeled SV40 DNA. SV40 DNA (lane 1) was reacted with 350 mM NaCl L1210 nuclear extract in the absence (lane 3) or presence of various concentrations of 8-MOC (0.1, 1, 10, 50, 100, 1000 µM in lines 4, 5, 6, 7, 8 and 9) or VP16 (50, 100 µM in lines 10 and 11). In lane 2 SV40 DNA reached with only 1000 M 8-MOC. Reactions were performed as in Fig. 7.

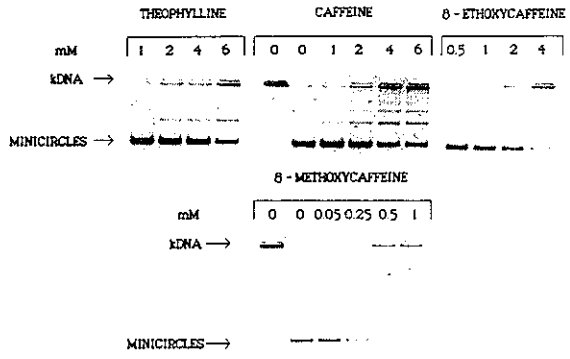

Fig. 9. - Effect of theophylline, caffeine, 8-ethoxycaffeine and 8-MOC on decatenation of kinetoplast DNA from *Chritidia fasciculata* by calf thymus DNA topoisomerase II. Decatenation of KDNA networks by the enzyme was followed by agarose gel electrophoresis. Decatenated minicircles, which are the products of the reaction, enter the gel, whereas the substrate networks remain at the origin. *Panel of 8-methoxycaffeine*: lane 1: KDNA networks control (no enzyme, no drug); lane 2: no drug; topoisomerase II present; lane 3-6: 8-MOC 0.05, 0.25, 0.5 and 1 mM respectively. *Panel of theophylline*: lane 1-4: theophylline 1, 2, 4 and 6 mM respectively. *Panel of caffeine*: lane 1: KDNA networks control (no enzime, no drug); lane 2: no drug; topoisomerase II present; lane 3-4: caffeine 1, 2, 4 and 6 mM respectively. *Panel of 8-ethoxycaffeine*: lane 1-4: 8-ethoxy caffeine 0.5, 1, 2 and 4 mM respectively.

classic antitumor topoisomerase II inhibitors and caffeine derivatives. Analogous results were obtained with the other caffeine derivatives (Fig. 9).

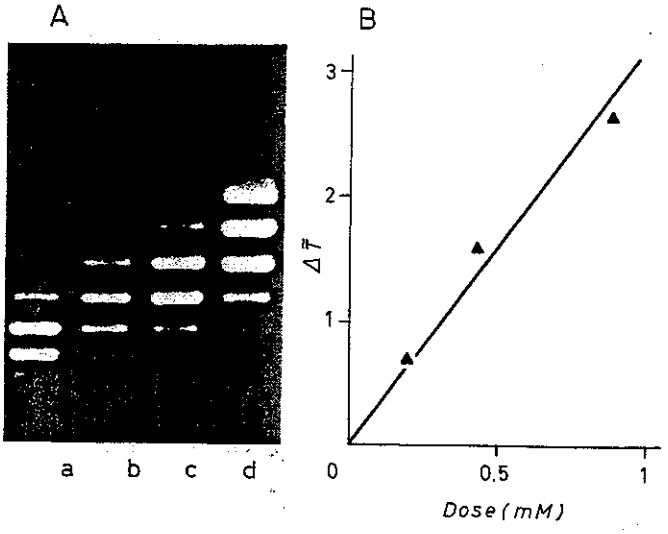

Fig. 10. - Unwinding measurement of closed circular DNA by 8-methoxycaffeine. *Panel a*: lane A: no addition of ligand; lanes B, C, D: 8-methoxycaffeine added at 0.22 mM, 0.45 mM, 0.89 mM. *Panel b*: quantification of the change in mean superhelicity of pAT153 DNA by 8-methoxycaffeine, obtained by calculating the shift of the center of the Gaussian as described.

DNA-unwinding experiments

Recently Pommier *et al.* [46] compared the DNA-unwinding effects of some 9-aminoacridine derivatives using a DNA topoisomerase I assay under reaction conditions that could be used to study drug-induced topoisomerase II inhibition. Drug-induced DNA unwinding is the most selective criterion of drug intercalation and could be used to detect and quantify this intercalation.

Since the majority of antitumor drugs that interfere with the strand-passing activity of DNA topoisomerase II are intercalators, we carried out studies to see if caffeine, 8-MOC, and 8-CC are able to bind to DNA, and if any such binding is intercalative. For this purpose we conducted an unwinding measurement using the plasmid pAT 153. We observed a change in the DNA mean linking number which was a function of the drug concentration. After quantification of the change in linking number introduced by caffeine, 8-MOC or 8-CC, we calculated that the concentration of 8-MOC needed to unwind of one superhelical turns pAT 153 DNA was 0.33 mM, (Fig. 10) for caffeine it was 2.70 mM and for 8-CC it was 0.50 mM. Thus at this point the question arises that the ratio of concentrations for intercalation and DNA breaks seen for MOPs are very different. For ellipticine and mAMSA this ratio is reasonably similar.

To sum up, our experiments, carried out in reconstituted systems gave negative or partially positive (only at very high concentration) results. These findings do not seem to be in favour of our working hypothesis that MOPs are able to inhibit DNA-topoisomerase II in mammalian cells.

Acknowledgements

The authors gratefully acknowledge the secretarial assistance of Miss G. Frigerio and also gratefully acknowledge the skilled, technical assistance of D. Piccini (graduate student, intership, Dpt. of Clinical and Experimental Oncology, University of Genoa).

This work was supported by Grant "Progetto Strategico Mutagenesi" No. 87.002502.74 and by Grant "Bilaterale Italia-USA" awarded by CNR, Rome, Italy and by grant EEC Contact Group, "Genetic effects of environmental chemicals", No. EV4V-036-I, Bruxelles, Belgium.

Review submitted on invitation by the Editorial Board of the *Annali*. Accepted for publication: October 1988.

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