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Biochemical Characterisation of Elsamicin and Other Coumarin-related Antitumour Agents as Potent Inhibitors of Human Topoisomerase II

Aurelio Lorico and Byron H. Long

Elsamicin (EM) is a recently discovered antitumour agent that is structurally related to several other compounds displaying anticancer activities, including chartreusin (CT), chrysomycin V (CV) and M (CM), gilvocarcin V (GV) and ravidomycin (RM). The biochemical events resulting in cytotoxicity for most of these compounds have not been clearly elucidated. There is some evidence that GV and CT bind to DNA and that GV is photosensitive, causing DNA damage. Therefore, we investigated the effects of these chemicals on DNA in cells and on pBR322 plasmid DNA. Using alkaline elution techniques, we found that all these compounds induced, to a different extent, DNA breakage in the human lung adenocarcinoma A549 cell line. In addition, all either bound to or intercalated into DNA, as indicated by their ability to alter the electrophoretic migration of DNA in agarose gels. Using the P4 unknotting assay, EM, CT, CV, CM, GV and RM were found to be potent inhibitors of the catalytic activity of topoisomerase II (topo II). Their potencies were compared with the known topo II inhibitors teniposide (VM-26) and doxorubicin (DX). EM was the most potent, with an IC_{50} of 0.4 μmol/l followed in order by CV, GV, and CT. VM-26 was the least potent with an IC_{50} of 15 μmol/l. It was concluded from these results that EM, GV, CV, CM and CT are capable of inhibiting topo II and that EM is the most potent inhibitor of topo II yet discovered.
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INTRODUCTION

ELSAMICIN (EM) IS a recently discovered coumarin-related compound displaying broad spectrum activities against *in vitro* and *in vivo* murine and human tumour models [1–3] and which has recently undergone phase I clinical trials under the name of elsamitrucin [4]. Its isolation from the culture broth of an unidentified actinomycete strain was first reported in 1986 [5]. Although it shares the same polycyclic aromatic hydrocarbon ring system (chartarin) as chartreusin (CT) [6, 7], the two compounds differ with respect to their dependent disaccharide moiety (Fig. 1). The two sugars found in CT are D-fucose and D-digitalose [7], whereas EM contains 2-amino-2, 6-dideoxy-3-*O*-methyl-D-galactose in place of D-fucose and 6-deoxy-3-C-methyl-D-galactose instead of D-digitalose (Fig. 1). The presence of the amino sugar makes EM significantly more water soluble than CT [3]. Interestingly, several other compounds with antitumour activity, previously discovered through anticancer screening programs, have similar (but not identical) aglycone ring systems to chartarin found in EM and CT. These include gilvocarcin V (GV), chrysomycin V (CV), chrysomycin M (CM) and ravidomycin (RM) (Fig. 1). While the latter compounds have one coumarin ring and a monosaccharide unit, EM and CT have two coumarin rings and a disaccharide unit. Also, novobiocin, which inhibits the ATPase function of prokaryotic [8] and, at higher doses, eukaryotic topoisomerase II (topo II) [9] has a coumarin ring and a pendent sugar (Fig. 1).

GV and gilvocarcin (GM) were discovered in the culture broth of *Streptomyces gilvotanareus* [10] and more recently in the culture broth of *Streptomyces anandii* [11]. GV showed both antibacterial and antitumour activity, while GM only showed weak antibacterial activity [10, 12]. The antitumour activity of GV has been attributed to the presence of a vinyl group instead of a methyl group in the 8 position [13, 14] (Fig. 1). The chrysomycins were first described in 1977 [15], and later, the producing streptomyces was isolated from a soil sample collected in Columbia and found to be similar to *Streptomyces albaduncus* ATCC-14698 [16]. We shall use the root name chrysomycin with V and M (Fig. 1) to refer to this class of congeners, as suggested by Matson *et al.* [16], with CM and CV representing methyl and vinyl congeners, respectively (Fig. 1). RM was isolated from the mycelium of *Streptomyces ravidus*, a bacterial strain found in a soil sample from Guatemala [17]. The literature is confusing with respect to the nomenclature for these compounds because of their almost simultaneous discoveries by different laboratories. Together, all of the compounds shown in Fig. 1 and their congeners can be grouped into a common class of compounds called coumarins.

All of the above compounds have shown antitumour activity both *in vitro* and *in vivo*, though to different extents [3, 12, 16–20]. For most of these compounds, the intracellular target(s) mediating the cytotoxic effect have not been well defined. Uesugi *et al.* have recently reported that EM induces selective DNA cleavage in the presence of dithiothreitol and ferrous sulphate [21]. CT inhibits RNA synthesis more than DNA synthesis and has the least effect on protein synthesis [19]. It also significantly inhibits DNA and RNA polymerases [19] and binds to DNA [22]. Furthermore, CT causes a single-strand scission into DNA

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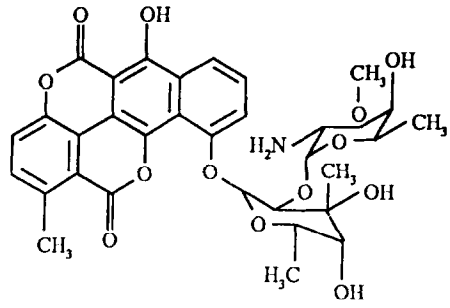
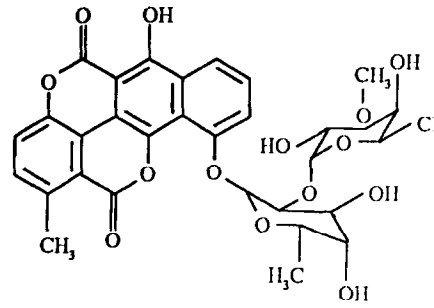
Dicoumarins (Chartarin ring)

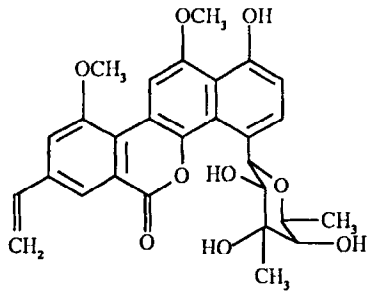


Elsamicin

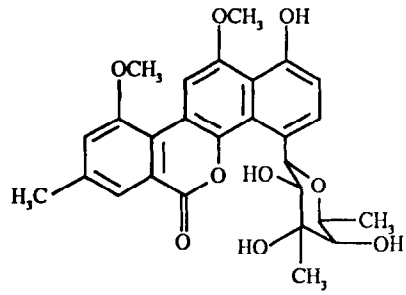


Chartreusin

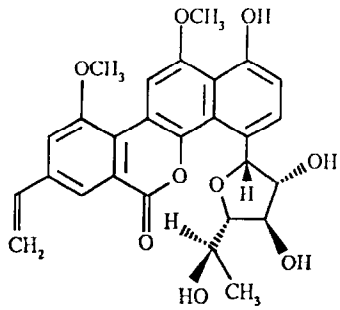
Coumarins



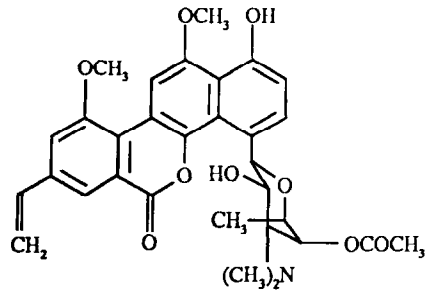
Chrysomycin V
(Chrysomycin A)
(Albacarcin V)
(Virenomycin V)



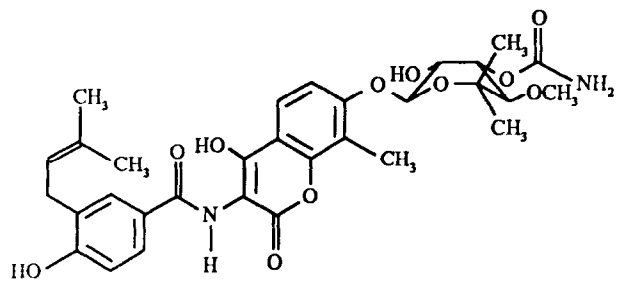
Chrysomycin M
(Chrysomycin B)
(Albacarcin M)
(Virenomycin M)



Gilvocarcin V
(Toromycin)
(Anandimycin)



Ravidomycin



Novobiocin

Fig. 1. Structures of similar dicoumarin and coumarin compounds.

in the presence of reducing agents such as dithiothreitol, sodium borohydride and ascorbic acid [23] and inhibits the relaxation of negatively supercoiled DNA by prokaryotic topoisomerase I. This latter effect seems to be due to the binding to DNA causing the alteration of tertiary structure [22]. The binding of CT correlates to the sequence of 3'GCG 5' on DNA stretch [22].

GV induces bacteriophage lambda in *E. coli* and causes single strand breaks (SSB) after photoactivation by low doses of visible light [13, 14]. When the exocyclic vinyl group is replaced with a methyl group (GM), the photochemical activity appears to be eliminated [24]. Also, in human cells photoactivated GV induces SSB and DNA–protein cross-links [25]. The potency of GV is attributed to its strong tendency to intercalate with DNA and its intense absorption of UV radiation [26]. Also, RM is a potent photosensitising, DNA-damaging agent [27]. Whether the observed *in vivo* antitumour activity of GV and RM is mediated by ambient light is not known.

The possibility exists that alternative mechanisms may account for the antitumour activities observed for these related compounds. In fact, the lack of systemic toxicity in animals may indicate either a selective distribution of GV or its activation at specific target tissues [13]. Since all these compounds are structurally related and since they all display antitumour activities, a better understanding of their mechanism of action may be obtained by structure–activity relationship studies. Until now, no effort has been made to directly compare most of these compounds. Therefore, we have analysed their effects on several biochemical and biological parameters both *in vitro* and on the A549 human lung carcinoma cell line. The results reported here demonstrate that all of the agents tested have antitumour activity, independent of the presence of a vinyl group, bind to DNA and inhibit human topo II. Also, EM is the most potent inhibitor of topo II.

MATERIALS AND METHODS

Cell culture, radioactive labelling and alkaline elution

Human lung adenocarcinoma cell line A549 was grown and maintained in McCoy's 5A tissue culture medium plus 10% heat-inactivated fetal bovine serum, penicillin and streptomycin and supplemented with pyruvate. For alkaline elution studies, drug-treated cells containing [^{14}C]DNA and cells containing [^{3}H]DNA were exposed on ice to 300 rads of gamma radiation to introduce random single strand DNA breaks. These were layered over polycarbonate filters (Nucleopore), washed twice with 10 μl of cold phosphate buffered saline, and lysed on the filters at room temperature by addition of SDS–EDTA lysis solution followed by lysis solution containing 0.5 mg/ml proteinase K (E. Merck, Darmstadt, Germany) [28]. DNA elutions were conducted at pH 12.1 with a pump flow rate of 40 μl/min.

Growth inhibition assay

The compounds were assayed for inhibition of A549 cell proliferation in 25 cm^2 flasks 5 to 9 days following a 1-h drug exposure in the dark, as previously described [29]. Cells surviving drug treatment were released from flasks with trypsin-ethylenediaminetetraacetic acid (EDTA), fixed with formaldehyde, and counted with a Coulter counter when the control flasks were generally 80% confluent. Counts for drug-treated cells were expressed as percentage of the average number of untreated control cells from four flasks for each experiment. At least three separate experiments, each based on five drug concentrations run in duplicate, were conducted for each drug. Generally, standard deviation of the six or more values converted to percentage of control value for each drug concentration did not exceed 10% and probit plots of the mean values vs. log concentration yielded correlation coefficients for linearity of greater than 0.95, as determined by linear regression analysis. A variance of plus or minus 10% in cell numbers expressed as percentage of control cells usually translated into a standard deviation for the IC_{50} value of less than 20% of the mean value.

DNA binding in vitro

To assay the binding activity of the different drugs we considered their ability to alter the electrophoretic migration of both supercoiled and nicked forms of pBR322 plasmid DNA in agarose gels. Reactions were carried out in reaction mixtures (20 μl each) containing Tris–HCl (50 mmol/l, pH 7.5), pBR 322 plasmid DNA (8 μg/ml) and drugs as indicated. After incubation at 37°C for 60 min, reactions were stopped with 5 μl of 50% glycerol, 20 mmol/l EDTA and 0.25% bromophenol blue, then analysed on a 0.9% agarose gel in Tris–HCl (80 mmol/l, pH 8.0), boric acid (40 mmol/l), and tetrasodium EDTA (2 mmol/l) (TBE buffer). To visualise better the shift in migration of DNA, no sodium dodecylsulphate (SDS) was added in the stopping buffer or in the gel.

Inhibition of DNA strand-passing activity of topo II

The strand-passing activity of topo II, partially purified from VACO-5 human colon carcinoma cells, was monitored by the P4 unknotting assay [30]. Reactions were initiated upon addition of 5 μl of human DNA topo II preparations to 20 μl reaction mixtures containing Tris (50 mmol/l, pH 7.5), NaCl (100 mmol/l), $MgCl_2$ (10 mmol/l), EDTA (0.5 mmol/l), bovine serum albumin (30 μg/ml), dithiothreitol (0.5 mmol/l), ATP (1 mmol/l), P4-knotted DNA (8 μg/ml), and drugs as indicated. After incubation at 37°C for 30 min, reactions were stopped with 5 μl of prewarmed stopping buffer (50% glycerol, 5% SDS, 20 mmol/l EDTA and 0.25% bromophenol blue) and then analysed on a 0.9% agarose gel in TBE buffer. Photographic negatives from ethidium bromide-stained gels were scanned by a LKB Ultrascan XL densitometer. The linear P4 band provided the arbitrary separation point between the knotted and the unknotted form. The topo II inhibition for each sample was calculated using the formula: $I = [(A-B)/(A-C)] \times 100$, where I is the inhibition activity (in %), A is the % of the unknotted P4 form in the sample incubated with topo II alone, B is the % of the unknotted P4 form in the sample incubated with topo II plus the investigational compound and C is the % of the unknotted P4 form in the control sample (no enzyme, no investigational compound).

RESULTS

Inhibition of cell proliferation

The potencies of EM, GV, CV, CM and CT as cytotoxic agents were compared with those of the known topo II inhibitors, doxorubicin (DX), VP-16, VM-26 and novobiocin (NB) (Table 1). CV and GV turned out to be the most potent on a molar basis, with an IC_{50} of 3 nmol/l and 36 nmol/l, respectively. The studies above were conducted in the presence of ambient light (see Materials and Methods). The IC_{50} for GV and CV increased to 8-fold and 4-fold, respectively, when care was taken to avoid light exposure during treatment, thus showing that photoactivation by ambient light plays a role in the cytostatic activity of these compounds. For EM, the IC_{50} did not change significantly when care was taken to avoid light exposure during treatment.

*Table 1. Median cytotoxic drug concentrations**

Drug	Abbreviation	Concentration(nmol/l) Light†	Dark†
Elsamicin	EM	3.4	3.6
Gilvocarcin V	GV	0.036	0.3
Chrysomycin V	CV	0.003	0.013
Chrysomycin M	CM	0.095	ND
Chartreusin	CT	95	ND
Doxorubicin	DX	0.67	ND
Etoposide	VP-16	8.4	ND
Teniposide	VM-26	0.5	ND
Novobiocin	NB	4600	ND

* A549 cells were exposed to the various drugs for 1 h and counted when the control cells were 80% confluent, as described in Materials and Methods. † Experiments were run in the presence or absence of ambient light. ND, not done.

*Table 2. Total strand DNA break formation produced in A549 cells by the different drugs**

Concentration of drug (μmol/l)	Breaks/10^8 nucleotides EM	GV	CV	CM	VM-26	CT
0.003		6.0				
0.01		14.5	3.5			
0.03		35	10.8		3.3	
0.1		136	35	7.1	11.7	
0.3				29	16.7	
1.0				96	28	7.2
3.0	54					39
10						

* A549 cells were exposed to the various drugs for 1 h in 60-cm diameter culture dishes then trypsinised with scraping and washed at 4°C and lysed at 24°C, and the DNA eluted overnight from polycarbonate filters at pH 12.1, as described in Materials and Methods.

DNA breakage produced in cells

Alkaline elution assays were used to compare DNA damage induced by the different drugs on A549 cells. Figure 2 shows typical dose–response curves of single-strand DNA breaks produced by CT, EM, GV and CV. The total DNA strand break formations by EM, GV, CV, CM, VM-26 and CT are described in Table 2. All these drugs clearly induce DNA breaks, though to different extents. The most active, on a molar basis, appear to be GV and CV (Table 2).

DNA binding in vitro

Li *et al.* and Gasparro *et al.* reported that CT [19] and GV [26] bind to or intercalate in DNA. Therefore, we investigated whether the drugs under study were also able to bind to purified DNA *in vitro*. As shown in Fig. 3, EM, GV, CV, CM, CT and DX bind to pBR322 plasmid DNA, producing a shift in migration of both supercoiled and relaxed forms of DNA, an indication of strong DNA binding or intercalation [32]. This shift is detectable for all drugs at the higher concentrations tested but is particularly evident for GV, CV and DX (Fig. 3).

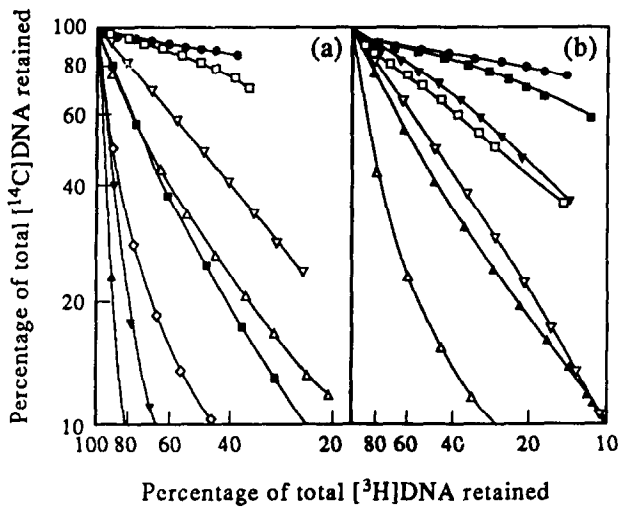


Fig. 2. DNA single-strand breaks in A549 cells. Cells containing ^{14}C-labelled DNA were incubated in the presence of various concentrations of EM, CT, GV and CV for 60 min at 37°C. Strand break frequency was determined at pH 12.1 by the high sensitivity alkaline elution method. Cells containing [^{3}H]DNA received 300 rads and were used as internal standards. (a): ●, no drug; □, 1 μmol/l CT; ▽, 3 μmol/l CT; △, 10 μmol/l CT; ■, 3 μmol/l EM; ◇, 30 μmol/l CT; ▼, 10 μmol/l EM; ▲, 30 μmol/l EM; (b): ●, no drug; ■, 10 nmol/l CV; ▼, 30 nmol/l CV; □, 10 nmol/l GV; ▽, 30 nmol/l GV; ▲, 100 nmol/l CV; △, 100 nmol/l GV.

Inhibition of DNA strand passing activity of topo II

Since all of these compounds induce DNA breaks in mammalian cells (Table 2) and strongly bind to pure DNA *in vitro*, it is possible that these drugs may inhibit topo II, as has been reported for the anthracyclines [33]. To investigate the effects of the different drugs on the catalytic activity of human DNA topo II, we monitored the strand-passing activity of the enzyme on knotted P4 DNA as substrate. CV, CM, EM, CT and GV strongly inhibited topo II activity in a dose-dependent manner (Fig. 4). These inhibition results were quantified, as described in Materials and Methods, and plotted vs. drug concentration (Fig. 5). EM and CV were the most potent, with an IC_{50} of 0.4 and 0.5 μmol/l, respectively, while 4′(9-acridinylamino) methanesulfon-m-anisidide, amsacrine (m-AMSA) and VM-26 were the least potent, with an IC_{50} of 11 μmol/l and 15 μmol/l respectively.

DISCUSSION

We have shown that a new class of antitumour compounds, characterised by the presence of a coumarin ring and including EM, GV, CV, CM and RM, displays a strong inhibitory activity on the catalytic activity of human topo II *in vitro* (as shown by the P4 unknotting assay) (Figs 4 and 5). Moreover, all of them have strong DNA binding ability, as demonstrated by the shift in electrophoretic mobility of both relaxed and supercoiled form of the pBR322 plasmid DNA (Fig. 3). Although GV and CV appear to be stronger DNA binders than EM, EM is the most potent *in vitro* topo II inhibitor yet known, and seems to be the most effective on intraperitoneally grown P388 leukaemia.

There is strong evidence that eukaryotic topo II is the target of many clinically important antitumour drugs such as anthracyclines, ellipticines, m-AMSA and the demethylepipodophyllotoxins VP-16 and VM-26 [33–35]. These drugs stabilise the enzyme intermediate formed between topo II and DNA, resulting in increased DNA excision, easily detectable by alkaline elution as SSB, double strand breaks (DSB) and DNA–protein crosslinks. A close correlation exists between the induction of such lesions and the cytotoxic effects of the drugs

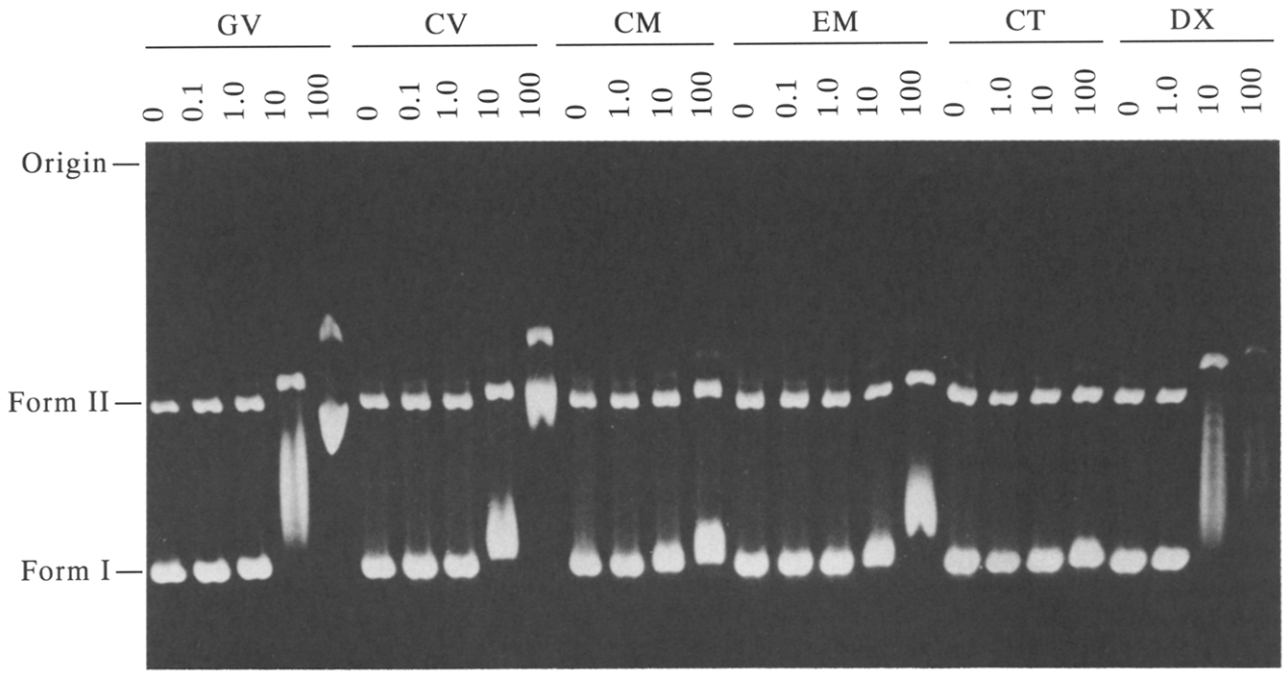


Fig. 3. Shift in electrophoretic mobility of both form I and form II of pBR322 plasmid DNA by the DNA binding of different doses of GV, CV, CM, EM, CT and DX in 0.9% agarose gel in the absence of SDS. Following electrophoresis, the gel was stained with 1 μg/ml ethidium bromide.

[36]. In many cases low sensitivity to topo II inhibitors is associated with a smaller number of drug-induced cleavable complexes and a lower cell topo II content [37]. Besides these quantitative differences, recent studies have shown there is a structurally modified topo II enzyme in some cancer cells which presumably makes these less susceptible to the action of topo II inhibitors [38]. The identification of other coumarin-containing compounds, in addition to novobiocin and the related antibiotic coumermycin [8], strengthens this class as potent topo II inhibitors and further supports the role of topo II as an important target for cancer chemotherapy.

The high *in vitro* cytotoxic potency of GV, CV and RM

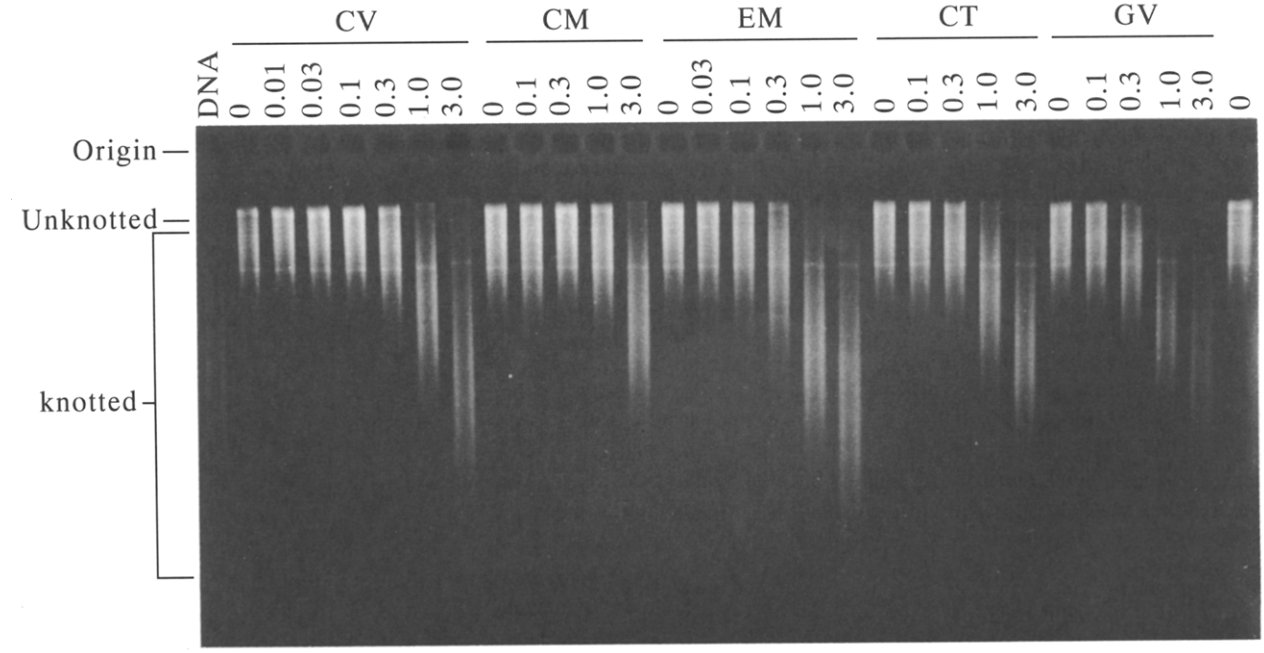


Fig. 4. Inhibition of the unknotting activity of partially purified human topo II by different doses of CV, CM, EM, CT and GV. P4 knotted DNA (8 μg/ml), topo II (1:100 dilution) and drugs (as indicated) were incubated in a 25 μl reaction mixture containing Tris (50 mmol/l, pH 7.5), NaCl (100 mmol/l), $MgCl_2$ (10 mmol/l), EDTA (0.5 mmol/l), bovine serum albumin (30 μg/ml), dithiothreitol (0.5 mmol/l), and ATP (1 mmol/l) for 30 min at 37°C. The enzyme was added last. Reactions were stopped with prewarmed stopping buffer and subjected to electrophoresis on a 0.9% agarose gel. The gel was stained with 1 μg/ml ethidium bromide.

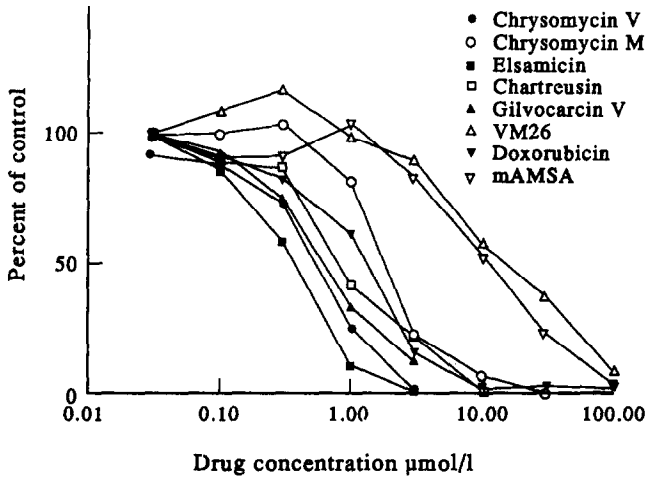


Fig. 5. Percent inhibition of the unknotting activity of partially purified human topo II by different doses of CV, CM, EM, CT, GV, VM-26, DX and m-AMSA. A negative of the agarose gel shown in Fig. 4 was scanned with a densitometer to quantify the inhibition of the DNA strand passing activity of topoisomerase by these compounds. Data are expressed as percentage of the unknotting activity of topo II in the absence of drug as described in detail in Materials and Methods. Standard deviations were always within 20% for each value.

has been related to photoactivatable DNA alkylating and/or breaking properties of these drugs conferred by the presence of the vinyl group in position 8 as evidenced by induction of prophage in lysogenic assays [10–14, 27], differential cytotoxicities of human cells *in vitro* [12, 27], and DNA breaks in cells *in vitro* [25, 26]. We have also found that a light-dependent cytotoxicity exists for A549 cells exposed to GV in the presence of ambient light for short periods of time (Table 1).

Whether the observed *in vivo* antitumour activity of GV, CV and RM [11, 16, 17] is mediated by ambient light is not known. It has been suggested that some light penetration to internal organs in animals as small as mice or that *in vivo* bioactivation of the agent may occur in order to account for their observed antitumour activities [13]. The lack of significant antitumour activity by GM has been used as evidence supporting the importance of the vinyl group for the antitumour activity of GV, CV and RM [12]. The evidence that CM has comparable antitumour activity to that of CV [15], and that the 8-ethyl analogue of RM is significantly more potent and displays greater antitumour activity than the parent compound [39], diminishes the importance of the vinyl group and suggests that other factors or modes of action must be involved in *in vivo* situations.

The light-dependent mechanism cannot account for the cytotoxicities and antitumour activities of EM and CT because, although these agents contain coumarin ring structures, they totally lack vinyl groups (Fig. 1). This statement is supported by our finding that in the absence or presence of light, the cytotoxic activity of EM on A549 human lung adenocarcinoma cells does not vary. We hypothesise that topo II inhibition by all of these compounds plays an important role in the antitumour activity *in vivo*, where photoactivation is less likely to occur, and that the very high cytotoxic potencies of GV and CV *in vitro* may be due to the photosensitive properties of these compounds. In fact, GV and CV are clearly the most potent compounds in terms of inhibiting the proliferation of A549 cells (Table 1), inducing DNA breaks (Table 2), and binding to DNA *in vitro* (Fig. 3), whereas CT is the least potent (Tables 1 and 2, Fig. 3). EM, which differs from CT only by the addition of a methyl group on the first sugar and substitution of an amino group for an hydroxyl group on the second sugar, has increased solubility [3], cytotoxicity, DNA break-inducing activity and DNA binding properties, relative to CT (Tables 1 and 2, Fig. 3).

EM and GV are the most potent inhibitors of topo II *in vitro* (Fig. 5), as well as the most potent antitumour agents [11, 16, 17, 31]. It is noteworthy that EM is much less effective than DX or GV at altering the migration of covalently closed, circular DNA in agarose gels (Fig. 3), yet has such a pronounced effect upon topo II inhibition (Fig. 5), suggesting either that no direct correlation exists between DNA binding and topoisomerase inhibition or that the type of DNA binding exhibited by EM is not comparable to that of DX. The mechanism by which these coumarin compounds inhibit topo II is not clear. Studies are in progress in our laboratories to clarify whether they accomplish topo II inhibition by interfering with the ATPase function of the enzyme, like novobiocin, or by stabilising topo II–DNA covalent complexes.

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Amplified *met* Gene Linked to Double Minutes in Human Glioblastoma

Bernd Wullich, Hans-Werner Müller, Ulrike Fischer, Klaus-Dieter Zang and Eckart Meese

The *met* proto-oncogene was found to be amplified in a human glioblastoma cell line (T3095) established from a glioblastoma multiform WHO grade IV. Amplification of epidermal growth factor receptor, transforming growth factor α and N-*myc* which have been described previously in glioblastoma were not observed in T3095. There was, however, an 8-fold *met* amplification. Giemsa-stained metaphases of T3095 cells revealed multiple (>5) double minutes (dmins) in the majority of cells. Following xenografting in nude mice there was a significant increase in the number and frequency of dmins. The increase in dmins correlates with the level of *met* amplification (50-fold), suggesting localisation of the amplified *met* on dmins. Here we report the first case of *met* amplification in glioblastoma. Correlation between *met* amplification and extrachromosomal elements (dmins) has not been reported previously.
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INTRODUCTION

GENE AMPLIFICATION, a process that increases gene copy numbers, has been demonstrated for a variety of different neoplasias [1]. Amplification of oncogenes is thought to contribute to a transformed phenotype via elevated expression of oncogenes. In some instances, detection of gene amplification within tumour cells has been shown to be of prognostic significance. Amplification and expression N-*myc* correlates with the stage of disease in patients with neuroblastoma [2–4]. In human breast cancer, amplification of *neu* gene is a significant predictor of both overall survival and relapse time, at least in a subset of patients [5]. In ovarian cancer, amplification of *erb*B2 is correlated with survival rates [6].

In glioblastoma the genes for epidermal growth factor receptor (EGFR), transforming growth factor α (TGFα), N-*myc*, *myc* and *gli* have been documented to be amplified [7–9]. Structural and numerical chromosome changes occur in approximately 75% of glioblastomas. Most striking is the gain of one or more entire