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Acknowledgements—We thank A. Plas for assistance with counterflow centrifugation, A. Pennings and C. Jacobs for help with flow cytometry, P. Linssen for HPLC measurements, A. Groeneveld for cooperation in the preliminary experiments, and Dr J.G.J. van de Winkel and Dr W.J.M. Tax, Division of Nephrology, University Hospital Nijmegen, The Netherlands, for providing antibodies. This work was supported by grant G4/87 from the University of Nijmegen Research Pool (UOP) and in part by the Maurits and Anna de Kock Foundation.

Eur J Cancer, Vol. 26, No. 5, pp. 586–589, 1990.
Printed in Great Britain

0277–5379/90$3.00 + 0.00

Potentiation by Phenylbisbenzimidazoles of Cytotoxicity of Anticancer Drugs Directed against Topoisomerase II

Graeme J. Finlay and Bruce C. Baguley

Analogues of the phenylbisbenzimidazole dye pibenzimol bind tightly to the minor groove of DNA. A clonogenic assay has been used to investigate the effects of these compounds on the cytotoxicity of the topoisomerase II directed anti-cancer drugs amsacrine, CI-921 (an amsacrine analogue), acridine carboxamide, etoposide and doxorubicin. Although pibenzimol itself was inactive, several of its analogues reduced the toxicity of etoposide, amsacrine and CI-921 towards a Lewis lung mouse tumour line at concentrations between 1 and 20 μmol/l. Doxorubicin cytotoxicity was unaffected, suggesting that this drug has a distinct mechanism of action. At concentrations below 1 μmol/l, some of these dyes potentiated the cytotoxicity of etoposide and CI-921 towards Lewis lung cells. Potentiation of CI-921 activity was also found with the human tumour lines HT29 (colon), SW620 (colon) and FME (melanoma). Novel treatments may arise from the potentiation of topoisomerase II directed cytotoxicity.
Eur J Cancer, Vol. 26, No. 5, pp. 586—589, 1990.

INTRODUCTION

TOPOISOMERASE II (topo II) is essential for DNA replication and is believed to be the target of anti-cancer drugs such as the epipodophyllotoxins, the anthracyclines and synthetic compounds, including amsacrine, its analogue CI-921, N-[2-(dimethylamino)ethyl]acridine-4-carboxamide (acridine carboxamide) and mitoxantrone [1, 2]. Previously we found that acridine carboxamide had the unusual property of inhibiting its own cytotoxic action at high concentration [3, 4]. We then showed that 9-aminoacridine and certain related DNA intercalators can chemoprotect against the cytotoxic effects of etoposide, amsacrine, CI-921 and acridine carboxamide both *in vitro* and *in vivo* [5].

We have used a series of Hoechst dyes with a clonogenic assay to investigate the modulation of cytotoxicity in cultured murine Lewis lung (LLTC) carcinoma cells. Phenylbisbenzimidazole dyes bind to DNA [6]. X-Ray crystallography reveals that pibenzimol (H33258) binds to the minor groove of double-stranded B-DNA and is selective for adenine–thymine rich regions [7]. This compound affects the chromosomes of growing cells, inhibiting the condensation of adenine–thymine rich regions [8] and it also inhibits the action of both topo I [9] and topo II [10] in cell-free systems. The effect of pibenzimol on murine leukaemia has prompted a phase I trial [11]. However, along with other minor groove DNA binding compounds [12], pibenzimol has not yet found a place in current cancer chemotherapy.

In this study we have investigated whether DNA minor groove binders can modulate the activity of topo II directed agents.

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MATERIALS AND METHODS

Chemicals

Phenylbisbenzimidazoles were provided by Dr H. Loewe (Hoechst AG, Frankfurt). Amsacrine and its 4-methyl-5-methylcarboxamide analogue CI-921 [13] were provided by Parke-Davis (Ann Arbor) as isethionate salts. We synthesized acridine carboxamide [3]. Stock solutions of these compounds were dissolved in 50% v/v aqueous ethanol to 1–5 mmol/l and stored at −20°C. Etoposide and doxorubicin were from Bristol Myers and Farmitalia Carlo Erba, respectively.

Cytotoxicity tests

A tissue culture adapted subline of LLTC cells [14] was obtained from Dr R.C. Jackson (Warner-Lambert, Ann Arbor). L1210 mouse leukaemia cells were obtained from Arthur D. Little Inc. (Boston). The human colon carcinoma cell lines HT-29 and SW620 were obtained from Dr J. Fogh and from the American Type Culture Collection, respectively. Human FME melanoma cells were from the laboratory of Dr K.M. Tveit, Norwegian Radium Hospital, Oslo.

Growth inhibition assays for LLTC cells were done in 96-well plates in α-MEM supplemented with 10% foetal bovine serum (FBS; Gibco), penicillin (100 U/ml) and streptomycin (100 μg/ml) [15]. Cultures were established with 10^3 cells per 150 μl. After 1 day, dyes were added and 2-fold dilutions made to encompass a 16-fold concentration range. Three days later, cultures were fixed and stained with methylene blue (2.5 g/l in 50% ethanol). Drug potency was expressed as the IC_{50}, the concentration of drug required to decrease cell growth to 50% of that in control (drug-free cultures). Growth inhibition assays for L1210 cells were done in 24-well plates in RPMI 1640 supplemented with 10% FBS, antibiotics as before and 2-mercaptoethanol (50 μmol/l) [16].

Clonogenic assays with LLTC cells and human cell lines were done as described [4, 5]. Cells were resuspended to 10^5 cells per ml in growth medium for exposure to bisbenzimidazole and anti-cancer drugs. Cells were preincubated with dyes for 1 h in a 37°C waterbath, after which topo II directed drugs were added for a further hour. Cells were collected by centrifugation, washed twice, resuspended in growth medium (containing 5% FBS) and 5 ml of different dilutions were plated in 60 mm dishes. After 9 (LLTC cells) or 10 (human cell lines) days, colonies were fixed and stained with methylene blue and those containing at least 50 cells were counted. Results are expressed as mean and S.E. of at least 2 replicates.

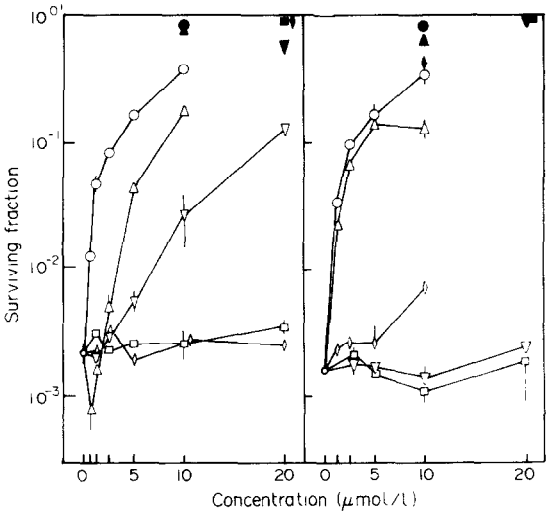

Fig. 2. Inhibition of cytotoxicity (LLTC cells) of topo II directed agents by phenylbenzimidazoles. After preincubation with dye, CI-291 2 μmol/l (open symbols) or an equivalent volume of solvent (shaded symbols) were added. Surviving fractions of cells exposed to CI-921 alone were 2.2×10^{-3} and 1.6×10^{-3} for the left and right hand panels, respectively. Bisbenzimidazoles: left hand panel— ○ = *p*-O-ethyl (H33342), △ = *p*-O-phenyl (H33377), ▽ = *p*-H, □ = *p*-NH_2 and ◇ = *p*-SO_2NH_2; right hand panel—○ = *p*-O-ethyl, △ = *p*-O-propyl (H33378), ▽ = *p*-OH (pibenzimol) □ = *m*-OH and ◇ = *p*-Cl.

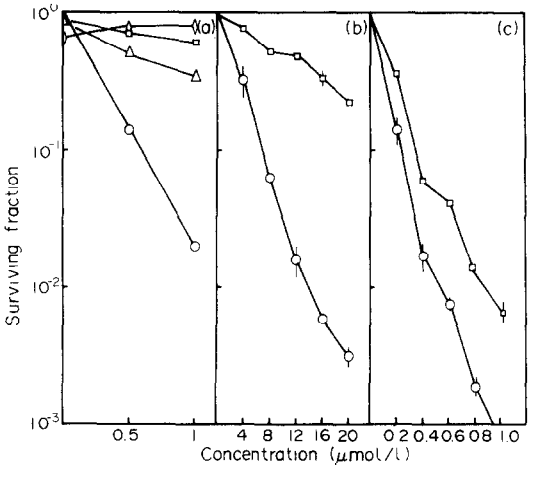

Fig. 1. Effect of H33342 (*p*-O-ethyl) on cytotoxicity of topo II directed agents. ○ = control and △ = 5, □ = 10 and ◇ = 15 μmol/l H33342. After preincubation, CI-921 (a), etoposide (b), or doxorubicin (c) were added.

Table 1. Cytotoxicities of phenylbenzimidazoles

Compound	Phenyl substitution	IC_{50} (nmol/l) LLTC	L1210
33187	H	1650 (50)	480
32993	*p*-NH_2	6300	1000
32985	*P*-methylpiperazinyl	190	330
33258 (pibenzimol)	*p*-OH	>10,000	1100 (68)
32021	*p*-OCH_3	—	130 (14)
33342	*p*-OCH_2CH_3	88 (12)	86 (22)
33378	*p*-$OCH_2CH_2CH_3$	160 (3)	95 (35)
333293	*p*-$O(CH_2)_3CH_3$	330 (85)	170 (78)
33377	*p*-O-phenyl	440 (100)	190
S769121	*p*-SO_2NH_2	9500	4500
32020	*p*-Cl	380 (120)	170
S785026	*m*-OH	5400	670

Mean (S.E.).

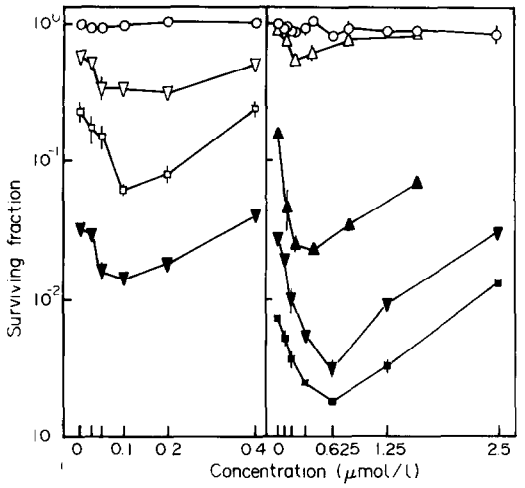

Fig. 3. Effect of H33342 and H33377 on cytotoxicity of CI-921 in LLTC cells. ○ = drug-free medium and △ = 0.05, ▽ = 0.1, □ = 0.3, ▲ = 0.5, ▼ = 1.0 and ■ = 2 μmol/l CI-391.

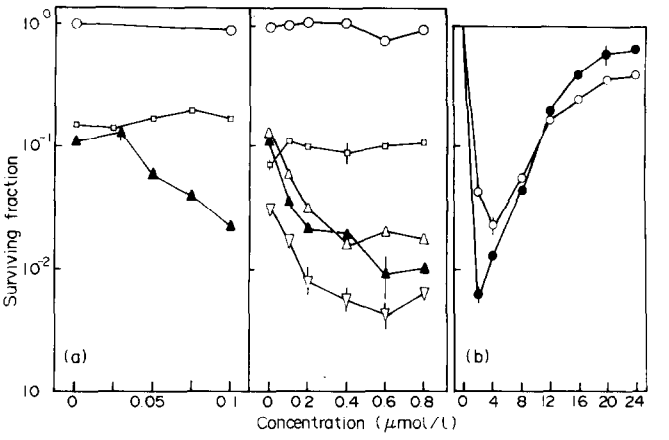

Fig. 5. Effect of H33342 and H33377 on cytotoxicity of anti-topo II agents. a. H33342 (left panel) or H33377 (right panel) at concentrations shown with LLTC cells. ○ = drug-free medium and △ = CI-921 0.5 μmol/l (final concentration), ▽ = acridine carboxamide 2 μmol/l, □ = doxorubicin 0.4 μmol/l, ▲ = or etoposide 8 μmol/l were added. b. Cells preincubated in control medium (○) or with H33342 (0.1 μmol/l, ●), followed by incubation with acridine carboxamide at concentrations shown.

RESULTS

H33342 antagonized the toxic effect of CI-921 on exponentially growing LLTC cells (Fig. 1A), effectively protected cells exposed to etoposide (Fig. 1B) but only slightly affected doxorubicin toxicity (Fig. 1C). When several analogues were compared, H33342 was the most potent chemoprotective agent; its homologues H33378, H33377 and the parent compound H33187 were less active (Fig. 2). These effects were observed at dye concentrations that were by themselves minimally cytotoxic. Some drugs, including pibenzimol, had no chemoprotective activity, even at high concentrations (Fig. 3). The rank order of potency was similar to the relative potencies of these compounds in growth inhibition assays (Table 1). L1210 and LLTC logarithmic IC_{50} values were also linearly correlated ($r = 0.75$).

H33377 slightly potentiated the cytotoxicity of CI-921 at low concentrations (625 nmol/l; Fig. 2 and data not shown), which prompted a more detailed study of the effect of H33342 and H33377 at low concentrations. When these compounds, at 1 μmol/l or lower, were added to cells exposed to CI-921, cytotoxicity was augmented (Fig. 3). The maximum potentiation obtained with H33342 was observed at lower concentrations that that with H33377, in agreement with differences in their IC_{50} values (Table 1) and chemoprotective (Fig. 3) activities. However, the maximum degree of potentiation was greater with H33377 than with H33342, with as much as a log of cell kill degree of magnitude compared with that obtained with CI-921 alone. H33377 also potentiated the cytotoxicity of CI-921 against HT29 colon, SW620 colon and FME melanoma cell lines (Fig. 4).

H33342 and H33377 at non-toxic concentrations potentiated the cytotoxicity of etoposide and acridine carboxamide towards LLTC cells (Fig. 5). H33342 100 nmol/l, when added to cells exposed to acridine carboxamide, promoted cytotoxicity at submaximally effective concentrations of acridine carboxamide, but accentuated the suppression of toxicity observed at higher carboxamide concentrations (Fig. 5B). Doxorubicin cytotoxicity was not potentiated by H33342 or H33377.

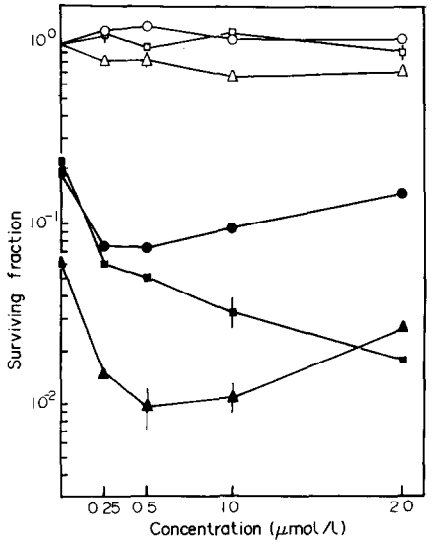

Fig. 4. Effect of H33377 on cytotoxicity of CI-921 against human cancer cell lines. HT-29 (○, ●), SW620 (△, ▲), and FME (□, ■) cells were preincubated with H33377 at concentrations on abscissa. CI-921 at 1 (SW620) or 2 (HT-29, FME) μmol/l (closed symbols) or equivalent dilution of solvent (open symbols) were added.

DISCUSSION

The inhibition of the *in vitro* cytotoxicity of some topo II directed anti-cancer drugs by some phenylbisbenzimidazole dyes resembles that observed for DNA binding agents such as ethidium [17] and 9-aminoacridine [5]. As with 9-aminoacridine, several of these dyes inhibited the cytotoxicity of etoposide, amsacrine, CI-921 and acridine carboxamide but not that of doxorubicin. However, in contrast to 9-aminoacridine, at least

two dyes also potentiated toxicity at low concentrations. If potentiation shows selectivity for tumour cells over normal cells, the therapeutic index might be increased.

The inhibition of cytotoxicity of anti-cancer acridine drugs and etoposide was paralleled by the potency of inhibition of cell growth in a continuous exposure IC_{50} assay. This association could be at least partly related to cellular uptake. Preliminary experiments (not shown) indicated that the active analogue H33342 (*p*-O-ethyl) is taken up by cells more so than pibenzimol (*p*-OH), which is inactive. The mechanism by which these dyes affect cytotoxicity has not yet been established. Simple DNA intercalators might inhibit production of the 'cleavable complex' form of topo II [12] and the DNA minor groove binders pibenzimol, diamidinophenylindole and distamycin inhibit the ability of amsacrine and teniposide to stimulate the formation of DNA–protein cross-links in isolated nuclei [10].

The potentiation by certain phenylbisdenzimidazoles of CI-921 cytotoxicity represents true synergy since the dye concentration required to elicit this effect is 10–100 times lower than that at which the compound is itself toxic. Nevertheless, such potentiation was observed only at CI-921 concentrations where a cytotoxic effect was apparent. H33377 consistently exerts greater potentiation than does H33342 on CI-921 cytotoxicity, despite the fact that it inhibits growth less than H33342 in IC_{50} assays. Thus it may be possible to use the substituents on the phenyl ring of the dye to differentiate chemoprotection from potentiation. Enhancement of etoposide cytotoxicity by H33342 and H33377 was also observed, while the lack of effect on doxorubicin cytotoxicity parallels that observed with simple intercalators [5] and implies that different mechanisms may operate for anthracyclines. Acridine carboxamide has a parabolic survival curve with increasing concentration [3, 4]. Addition of H33342 shifted the curve towards lower drug concentration, suggesting effects on both potentiation and chemoprotection.

The mechanism of potentiation of cytotoxicity by these dyes is not understood. Distamycin at a low concentration stimulated the relaxation of supercoiled DNA by topoisomerase I in a cell-free system by up to 38% [9] and also slightly stimulated the teniposide-induced formation of DNA–protein cross-links in L1210 cell nuclei [10]. We have been unable to demonstrate any effect of distamycin on the cytotoxicity of CI-921 in the LLTC system at distamycin concentrations between 0.16 and 45 μmol/l (M. Chin and G.J. Finlay, unpublished). It is possible that the hydrophobic group (ethyl or phenyl) attached to H33342 or H33377 plays a role in the stabilization of the 'cleavable complex' induced by topo II directed agents.

It is clear from the results with HT29 colon, SW620 colon and FME melanoma cell lines that H33377 can potentiate the cytotoxicity of some topo II agents in human cell lines as well as in the Lewis lung line, which suggests a clinical application. The problems of designing a potentiator that is both delivered to the tumour *in vivo* at the required concentration and selective for tumour cells have yet to be investigated. Optimal substitution of the phenylbisbenzimidazole structure may give rise to even more effective compounds. The best compound so far studied (H33377) effectively halves the concentration of CI-921 required for a given amount of cell killing. Potentiation of topo II directed cytotoxicity opens up novel treatment strategies.

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Acknowledgements—We thank Dr H. Loewe for providing the dyes. We also thank Bill Denny for helpful comments and Lynden Hull for preparing the manuscript. This study was supported by the Auckland Division of the Cancer Society of New Zealand, the Medical Research Council of New Zealand and a Warner-Lambert Laboratory Fellowship.