Chemical and Biological Explorations of the Family of CC-1065 and the Duocarmycin natural products

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Key words (8)

CC-1065, Duocarmycin SA, adozelesin, CBI, CPI, DNA minor groove binders (MGBs), adenine N3 alkylation, prodrugs

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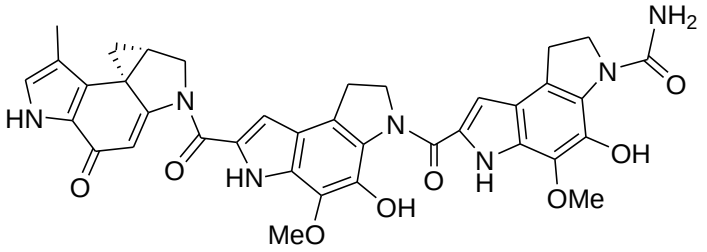
ABSTRACT

CC-1065, the duocarmycins and yatakemycin are members of a family of ultrapotent antitumour antibiotics that have been the subject of extensive investigations due to their mode of action and potential in the design of new anticancer therapeutics. The natural products and their analogues exert their effects through a sequence selective alkylation of duplex DNA in the minor groove at the N3 of adenine. An understanding of their structure and its effect on biological activity has been derived through chemical synthesis and has also generated new potential lead compounds. These studies form the first section of the review. The desire to progress these compounds to clinic has also led to studies of bioconjugation and prodrug formation and this is discussed in the second section of the review. The combination of synthesis with key biological experiments is a powerful tool to define the requirements for the development of natural products as potential therapeutic agents. The studies described herein form an excellent paradigm for the study and development of other natural products.

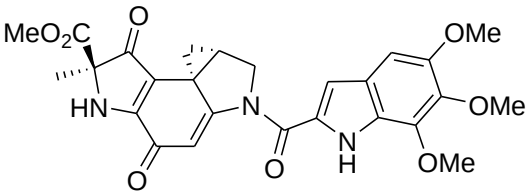
INVESTIGATIONS OF CC-1065, THE DUOCARMYCINS AND SYNTHETIC ANALOGUES FOR ANTICANCER PROPERTIES

(+)-CC-1065 (**1**) and the duocarmycins (**2**-**9**) (Fig. **1**) are natural products isolated from the culture broth of *Streptomyces species*, which have been shown to exert ultra-potent activity against cultured cancer cells and in experimental animals [1-3]. More recently, (+)-yatakemycin (**10**) has been isolated from *Streptomyces sp*. and represents the most potent member of this class of natural products [4]. The biological activity of these natural products is related to a characteristic sequence-selective alkylation of adenine N3 in AT-rich sites by the least substituted carbon of the activated cyclopropane (Fig. **2**) [5, 6]. This minor groove binding is thought to initiate a cascade of cellular events leading to apoptosis as observed for the duocarmycins [7]. The broad spectrum of antitumour activity and high potency of (+)-CC-1065 supported its potential as a clinical candidate, unfortunately a delayed death profile due to catastrophic hepatotoxicity was observed in experimental animals [8]. It has been speculated that this delayed toxicity is related to the degree of reversibility of the DNA alkylation and consequently to the extent of non-covalent binding stabilization of the reversible DNA adduct [9]. (+)-Duocarmycin SA (DSA, DUMSA, **3**) has also been found to be too toxic for systemic use, although it is devoid of hepatotoxicity [10]. The failure of the clinical progression of CC-1065 and the duocarmycins has prompted many research groups to use these natural products as leads for the design of novel, and more efficacious agents with reduced adverse toxicity. Since their first isolation, a number of total syntheses of these natural products have been extensively reported and reviewed [1, 2]. Additionally, numerous investigations have been directed towards the synthesis of authentic alkylating pharmacophores and analogues containing deep-seated structural modifications, defining the relationship between structure, functional reactivity and biological properties. Exciting insights into both the synthetic and mechanistic aspects of this family of natural products have

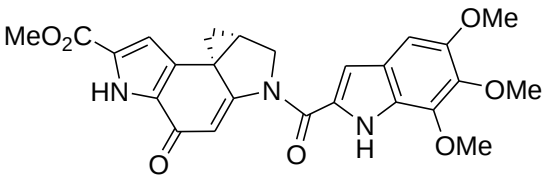
previously been described [1, 2] and the field has recently been further reviewed [11-14]. This review serves to update the field over the past decade and will describe novel directions in regard to chemical and biological investigations of synthetic analogues and prodrug derivatives, which aim to increase tumour selectivity and prepare agents for clinical progression. (+)-Yatakemycin (**10**) and related sandwiched small molecules will not be described here, as a recent account of their discovery has already been reported [15].



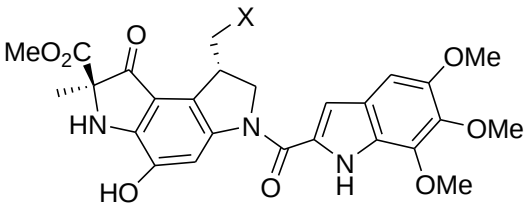
(+)-CC-1065 (**1**)



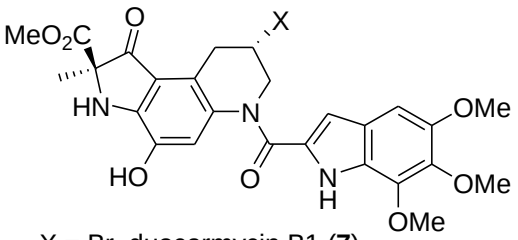
(+)-duocarmycin A (**2**)



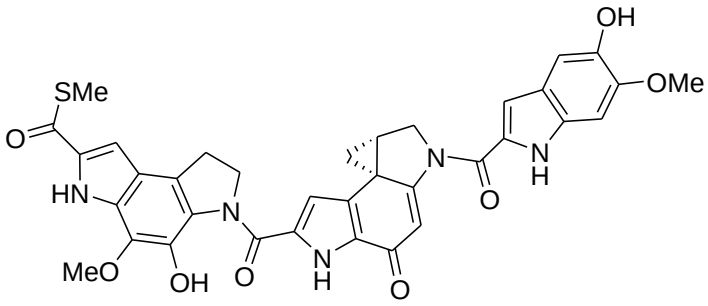
(+)-duocarmycin SA (**3**)



X = Br, duocarmycin B2 (**4**)
X = Cl, duocarmycin C2 (**5**)
X = OH, duocarmycin D2 (**6**)



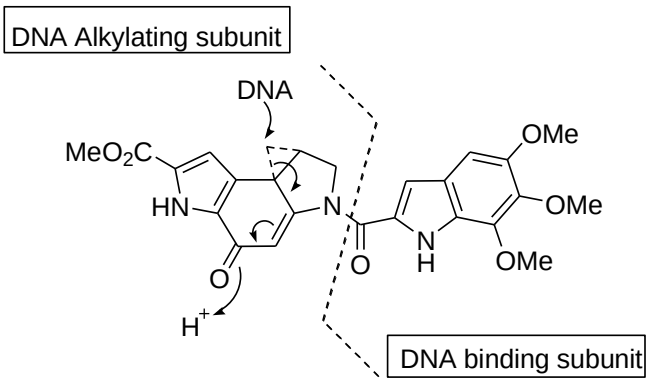
X = Br, duocarmycin B1 (**7**)
X = Cl, duocarmycin C1 (**8**)
X = OH, duocarmycin D1 (**9**)



(+)-Yatakemycin (**10**)

Figure (**1**). Structures of (+)-CC-1065, (+)-duocarmycins and (+)-yatakemycin

Due to the intense potency, unique mechanism of action and broad range of activity of the duocarmycins, extensive efforts have been made during the past two decades to find analogues that retain their potency and antitumour activity with potential for clinical progression. Early studies of the acid-catalyzed activation of the DNA alkylation reaction revealed a direct relationship between stability towards solvolysis and biological potency, and this relationship has proved to be general with both simple and full analogues of the natural products (for an excellent summary of these studies see ref. [2]). Agents containing the natural (CPI, DSA) or modified (CI, CBI, CBQ, CFI) alkylation subunits (Fig. **3**) attached to the same DNA binding subunits have been found to alkylate DNA at the same sites; the distinctions between the agents lie in the greater selectivity among the available sites with chemically more stable agents (DSA > CBI > CPI > CBQ > CI).



(+)-duocarmycin SA (**3**)

Figure (**2**). Mechanism of DNA alkylation by the duocarmycins

From thorough examination of the structure-activity relationships (SARs), a parabolic correlation between stability and biological activity has been established [16-18]. Compounds should possess sufficient stability to enter the cell, yet maintain sufficient reactivity to effectively alkylate the DNA upon reaching it [17]. The SARs served to locate the optimal

balance point of stability and reactivity that may be used for future design of related analogues. The alkylation subunits of DSA (**3**) and yatakemycin (**10**) lie at the peak of stability/reactivity whereas the CC-1065 (**1**) and duocarmycin A (**2**) alkylation subunits fall significantly to the over-reactive side of this optimal position [17, 18]. In the following sections, highlights from research on various pharmacophores with alkylating potential are presented, leading to a discussion on prodrug approaches that are more likely to lead to successful candidates for clinical progression.

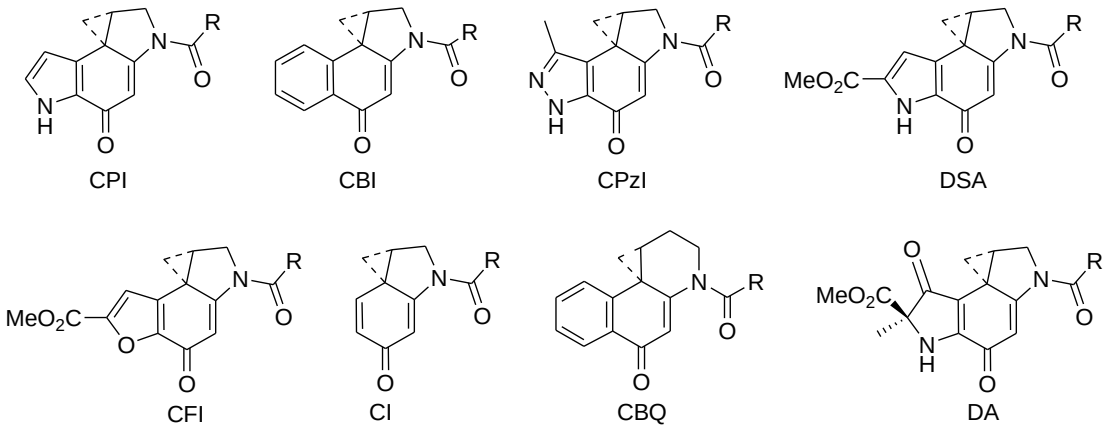


Figure (**3**). Structures of different alkylating subunits

SAR EXPLORATION ON DNA ALKYLATING PHARMACOPHORES

1,2,7,7a-tetrahydrocyclopropa[*c*]indol-4-one (CI)

The first report of the CI subunit was disclosed in the early 1980s [19]. This drastically truncated small molecule proved to be an exceptionally reactive electrophile, with a DNA alkylation pattern similar to duocarmycin-based agents, albeit with lower efficiency. Detailed studies of the CI analogues including introduction of substituents to the phenyl ring

(Fig. **4**), modification of the minor groove targeting moiety R, and variation of the leaving group X of the *seco*-CI precursor have been reviewed previously [1].

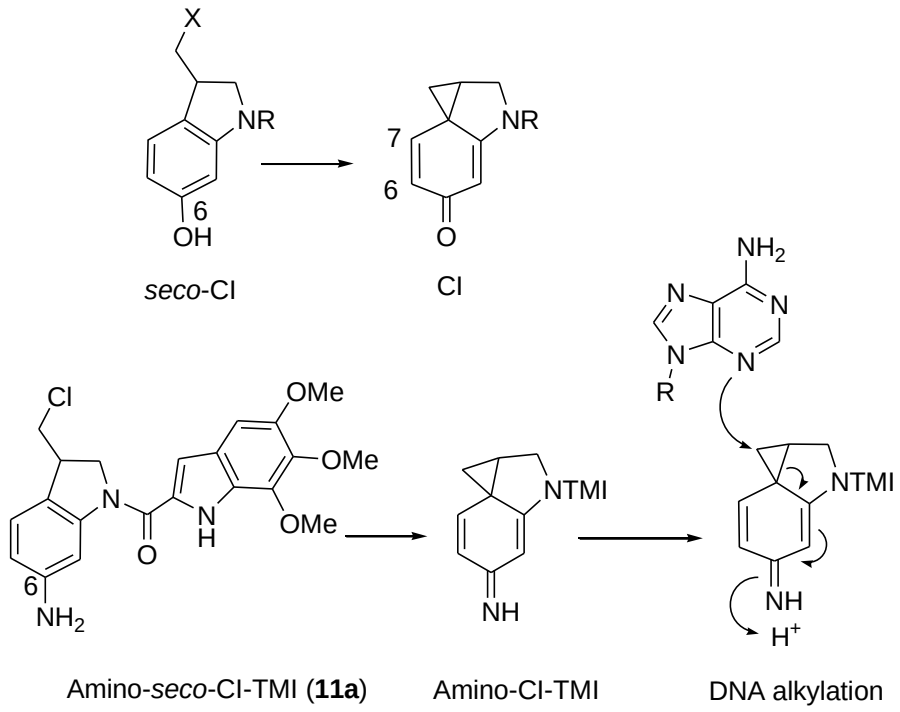
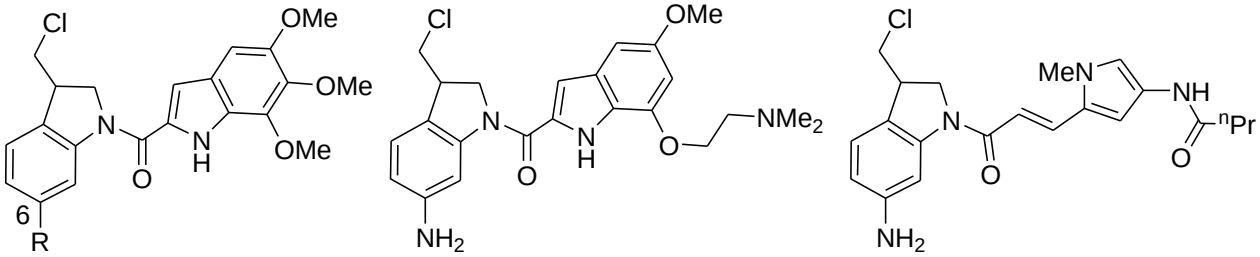


Figure (**4**). Spirocyclisation and alkylation mechanisms of CI-type subunits.

Modifications to the C-6 phenol of *seco*-CI (Fig. **4**, *seco*-CI numbering) have not been extensively investigated. Studies with ethers revealed that the free phenol is not obligatory for DNA alkylation; both the alkylating ability and cytotoxicity relates to the electron-donating ability of the C-6 substituent (*seco*-CI numbering), while complete removal of the substituent led to a complete loss of activity [20]. This observation led to the exploration of several sub-classes of amino-*seco* alkylating pharmacophores based on the CI moiety as potential reduction metabolites derived from prodrugs designed to be activated under the hypoxic conditions of solid tumours [21]. Assessment of these compounds as cytotoxins is reported here in order to compare their reactivity with the parent natural products, but will be put further into context under the description of prodrug approaches. Initially, amino and thio *seco*-CI alkylating pharmacophores were investigated and whereas the latter were surprisingly

inactive (IC_{50} > 50-200 μM) under the conditions investigated, the amino-*seco* CIs (**11**) were shown to produce a moderate cytotoxic response, albeit notably less potent than *seco*-CI itself [22]. Subsequent to the preliminary investigation, a wider series of amino-*seco* compounds were synthesized and shown to display similar *in vitro* potencies (Fig. **5**) [23]. In spite of this loss of potency, covalent binding to DNA was still observed at the adenine N3 position [21, 23, 24]. It is tempting to assume the amino-*seco* CIs spirocyclise in a similar fashion to hydroxyl-*seco*-CI (Fig. **4**), and indeed comparison of hypersensitivity factors suggests a similar mechanism of action [22].



11a R = NH_2; IC_{50} = 0.27 μM
11b R = NHMe; IC_{50} = 0.11 μM
11c R = NMe_2; IC_{50} = 0.11 μM
***seco*-CI** R = OH; IC_{50} = 0.0022 μM

11d IC_{50} = 0.09 μM

11e IC_{50} = 0.91 μM

Figure (**5**) Cytotoxicity of racemic *seco*-amino-CI analogues against EMT6 murine mammary carcinoma cells

A novel class of achiral *seco*-CI analogues containing the 4-hydroxyphenethyl halide functionality instead of the indoline ring (as exemplified by **12**) that are able to generate a spiro[2,5]cyclopropanecyclohexadienone **13** with capacity to alkylate DNA have been reported [25, 26]. These agents react with adenine-N3 with an alkylation pattern similar to CC-1065 (Fig. **6**) and showed low micromolar cytotoxicity in the NCI panel of 60 human tumour cell lines, with a unique pattern of activity that may merit further investigation. In particular, compound **12** was very active against melanoma cell lines. A subsequent *in vivo* study with the UACC-257 human melanoma cell line showed activity, with increased mean

time for tumour weight doubling of 27.7 days (control 15.8 days) and no bone marrow toxicity [25].

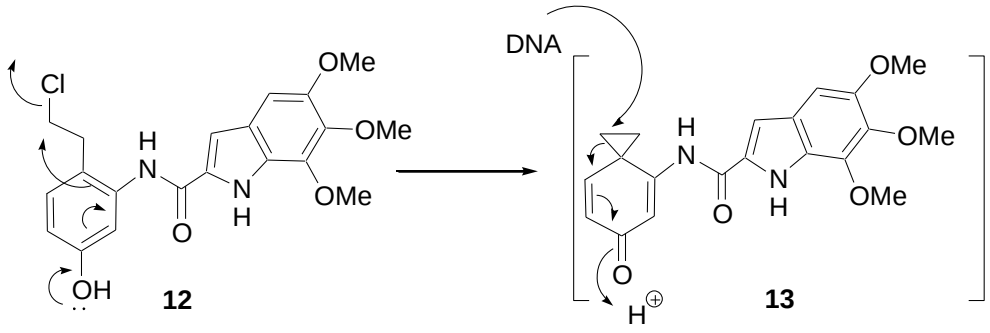


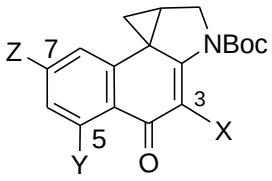
Figure (**6**). Achiral CI-TMI analogue

1,2,9,9a-tetrahydrocyclopropa[*c*]benz[*e*]indol-4-one (CBI)

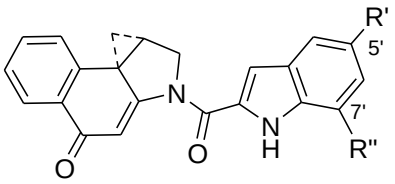
CBI-based analogues are synthetically more accessible than the natural products, are chemically more stable and retain potent biological activity [2, 27-41]. The CBI subunit has found significant applications in prodrug therapy and work in this area is discussed later in this review. Recent investigations have included functionalization of the C3 [31], C5 [42] and C7 positions [43], and of the DNA minor groove binding subunit [27, 29] (Fig. **7**). The solvolytic reactivity of the C3-substituted halogen series (**14a-e**) followed the trend of increasing reactivity with diminished vinylogous amide stabilization (I > Br > Cl > F > H) and their cytotoxic activity (against L1210 cell line) is remarkably less than that of the unsubstituted parent *N*-Boc-CBI itself [31]. The introduction of an ester group at C5 (**14g**) decreased the rate of DNA alkylation while maintaining the DNA alkylation selectivity. The C5 ester does not bind in the minor groove of DNA [42]. In contrast, analogues bearing C7 substituents (**14i** –OMe [43] and (**14j** -CN [44]) had an increased rate of DNA alkylation, which was attributed to the fact that the C7-cyano substituent increases the rigid length of the

alkylation subunit, leading to an extended degree of DNA binding-induced twist, thus aiding the activation of the cyclopropane ring towards nucleophilic attack.

The CBI-analogues bearing different DNA binding indoles (**15**, Fig. **7**) were assessed for their cytotoxicity against L1210 cells and it was found that C5' substituents (**15a-f**) had a pronounced effect on the biological activity; the cytotoxicity was largely insensitive to the electronic character of the C5' substituent, but sensitive to its size, shape and rigidity [29]. Substituents at C7' (**15g-i)** had little effect on cytotoxicity.



(+)-14a	X = H;	IC_{50} = 0.08 μM	**(-)-14a** IC_{50} = 0.9 μM
(-)-14b	X = F;	IC_{50} = 0.2 μM	**(+)-14b** IC_{50} = 3.7 μM
(+)-14c	X = Cl;	IC_{50} = 2.1μM	**(-)-14c** IC_{50} = 3.9 μM
(+)-14d	X = Br;	IC_{50} = 1.4 μM	**(-)-14d** IC_{50} = 4.7 μM
(+)-14e	X = I;	IC_{50} = 1.2 μM	**(-)-14e** IC_{50} = 4.2 μM
(+)-14f	X = CH_3;	IC_{50} = 3.1 μM	**(-)-14f** IC_{50} = 7.4 μM
(+)-14g	Y =CO_2Me;	IC_{50} = 11 μM	**(-)-14g** IC_{50} = 13 μM
(+)-14h	Y = CH_2OH;	IC_{50} = 0.09 μM	**(-)-14g** IC_{50} = 0.2 μM
(+)-14i	Z = CN;	IC_{50} = 20 nM	**(-)-14i** IC_{50} = 80 μM
(+)-14j	Z = OMe;	IC_{50} = 90nM	**(-)-14j** IC_{50} =200 μM
(natural enantiomer)			(unnatural)



15a R' = CN;	IC_{50} = 30 pM
15b R' = OEt;	IC_{50} = 10 pM
15c R' = COMe;	IC_{50} = 2 pM
15d R' = H;	IC_{50} = 2700 pM
15e R' = C≡CH;	IC_{50} = 300 pM
15f R' = C≡CMe;	IC_{50} = 1000 pM
15g R" = COMe;	IC_{50} = 300 pM
15h R" =CN;	IC_{50} = 300 pM
15i R" = OMe;	IC_{50} = 300 pM

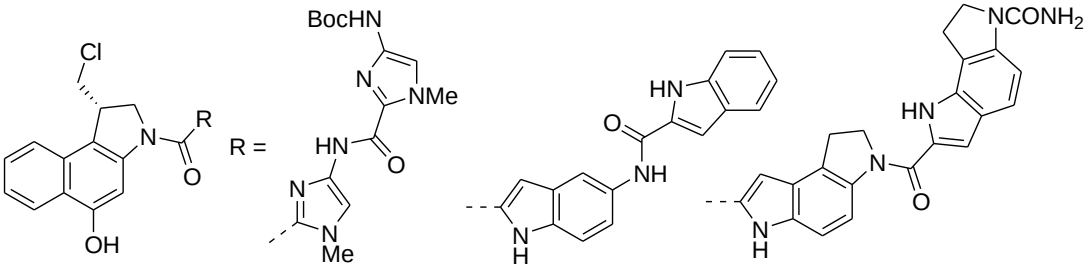
(only natural enantiomers prepared)

Figure (**7**). CBI analogues (IC_{50} data obtained from screening against L1210). All substituents (X, Y, Z, R', R'') are H unless otherwise indicated.

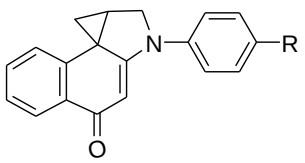
More complex variations on the minor groove binding right hand subunit have been investigated. Analogues containing dimers of monocyclic, bicyclic and tricyclic heteroaromatics such as **16** showed a general trend of increasing DNA binding rate and efficiency in the order tricyclic (**16c**) > bicyclic (**16b**) > monocyclic (**16a**) heteroaromatic subunits with parallel changes in cytotoxicity but no effect on sequence specificity [28].

These results highlight the importance of the minor groove binding domain in catalyzing the DNA alkylation reaction, and contain important information for the design of further analogues.

A series of *N*-aryl (**17**) and *N*-alkenyl CBI (**18**) derivatives [17, 18] showed remarkable stability while displaying a well-defined SAR. Electron-withdrawing substituents R enhance solvolysis rate and biological activity (eg. **17e**, **18b**), and electron-donating substituents have the opposite effect (eg **17b**, **18d**). Replacement of the amide linker with an alkene as in **18** reduces the cytotoxic potency by approximately 1000-fold [17]. Although the unnatural enantiomers are less biologically active than the natural, both natural and unnatural enantiomers fit the same SAR relationship. These data played an important role in the establishment of the parabolic stability/toxicity relationship described by Boger's group.

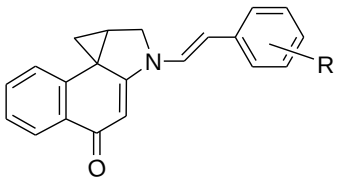


16a IC_{50} >1000 pM **16b** IC_{50} 19 pM **16c** IC_{50} 5 pM



(+)-17a R = NMe_2; IC_{50} = 3800 nM
(-)-17b R = OMe; IC_{50} = 3200 nM
(+)-17c R = H; IC_{50} = 1100 nM
(+)-17d R = CO_2CH_3; IC_{50} = 330 nM
(-)-17e R = NO_2; IC_{50} = 40 nM
(natural enantiomer)

(-)-17a IC_{50} > 10000 nM
(+)-17b IC_{50} = 18000 nM
(-)-17c IC_{50} = 5000 nM
(-)-17d IC_{50} = 2500 nM
(+)-17e IC_{50} = 330 nM
(unnatural)

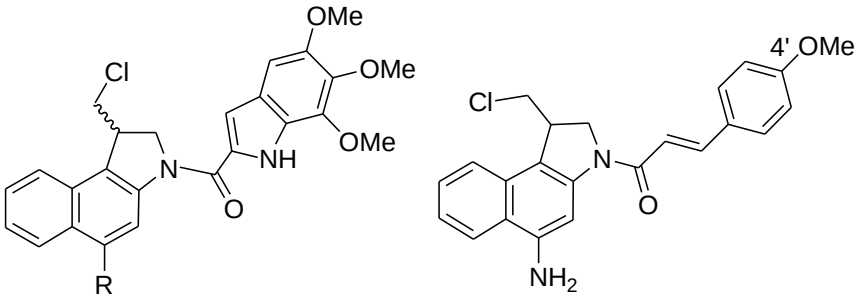


(+)-18a R = H; IC_{50} = 40 nM
(+)-18b R = NO_2; IC_{50} = 10 nM
(+)-18c R = 3,4,5-OCH_3; IC_{50} = 140 nM
(-)- 18d R = 2,3,4-OCH_3; IC_{50} = 380 nM
(natural enantiomer)

(-)-18a $IC5_0$ = 700 nM
(-)-18b IC_{50} = 420 nM
(-)-18c IC_{50} = 680 nM
(+)-18d IC_{50} = 340 nM
(unnatural)

Figure (**8**). CBI analogues bearing aryl and alkenyl DNA-binding subunits. IC_{50} data obtained from L1210 cell line.

The synthesis and biological evaluation of racemic and enantiomerically pure amino-*seco*-CBI compounds (**19**, Fig. **9**) have been described. In contrast to the CI class (e.g. **11**), the amino-*seco*-CBI-TMI compounds showed equipotent activity with the corresponding *seco*-phenol compounds (Fig. **9**). Structure-activity relationships for several other analogues have also been described with the 4'-OMe-cinnamoyl derivative (**20**) proving to be the most active with comparable potency to that of parent CBI-TMI [30, 41, 45, 46].

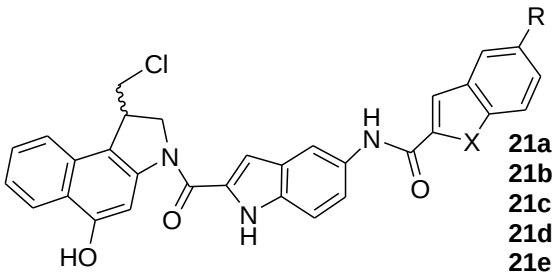


(±)-19a R = NH_2; IC_{50} = 0.46 nM
(+)-19a R = NH_2; IC_{50} = 0.21 nM
(-)-19a R = NH_2; IC_{50} = 14 nM
(±)-19b R = NHMe; IC_{50} = 0.17 nM
(±)-19c R = NMe_2; IC_{50} = 5.6 nM
R = OH; IC_{50} = 0.21 nM

(±)-20 IC_{50} = 0.09 nM

Figure (**9**). Amino-CBI analogues. Cytotoxicity assessed in the AA8 CHO cell line

Explorations of CBI analogues with an extended minor groove binding moiety in an effort to mimic (+)-CC-1065 have been reported. Compounds (**21a**-**g**) (Fig. **10**) were tested *in vitro* against U937 and L1210 leukemia cells and *in vivo* in L1210 tumours in mice with **21a** being chosen for further investigation. It showed good activity across the NCI 60 tumour cell line panel and, in an *in vivo* experiment, increased the lifespan of mice bearing L1210 tumours by 107% with low myelosuppression as a side effect. It was also effective against B16 melanoma tumours. Unfortunately, further investigations revealed a delayed toxicity profile similar to CC-1065, which was attributed to the presence of a methyl group in the C5-$COCH_3$ substituent since **21e-g** did not cause delayed death [40, 47].



21a X = NH, R = $NHCOCH_3$; IC_{50} = 1.12 nM (U937 0.4 nM)
21b X = O, R = $NHCOCH_3$; IC_{50} = 0.65 nM
21c X = NH, R = NHCOBu; IC_{50} = 0.008 nM (U937)
21d X = NH, R = OCH_3; IC_{50} = 5.7 nM
21e X = O, R = NO_2; IC_{50} = 0.44nM
21f: X = NH, R = F; IC_{50} = 0.29 nM
21g: X = O, R = F; IC_{50} = 0.85 nM

Figure (**10**). CBI based CC-1065 analogues. Activity against L1210 cells unless otherwise indicated

Investigations on the achiral *seco*-CBI class of agent led to the development of compounds (**22a**-**g**, Fig. **11**) that were more active than the corresponding analogues (**12**) in the CI series described above (Fig. **6**) [48]. Compounds with a good leaving group X (e.g. **22a, d**-**g**) were found to be over 100-fold more cytotoxic against leukemia L1210 cells than analogues bearing a poor leaving group (**22c**). The achiral 4-amino-1-(2-chloro-ethyl)-naphthalen-2-yl-TMI (**22a**) alkylated DNA in a sequence selective manner and had substantial *in vivo* antitumour activity against 12 cell lines in the NCI hollow fibre assay, and against MDA-MB-435, SF-295, OVCAR-3 and NCI-H522 subcutaneous tumours when administered p.o. or i.v. [48].

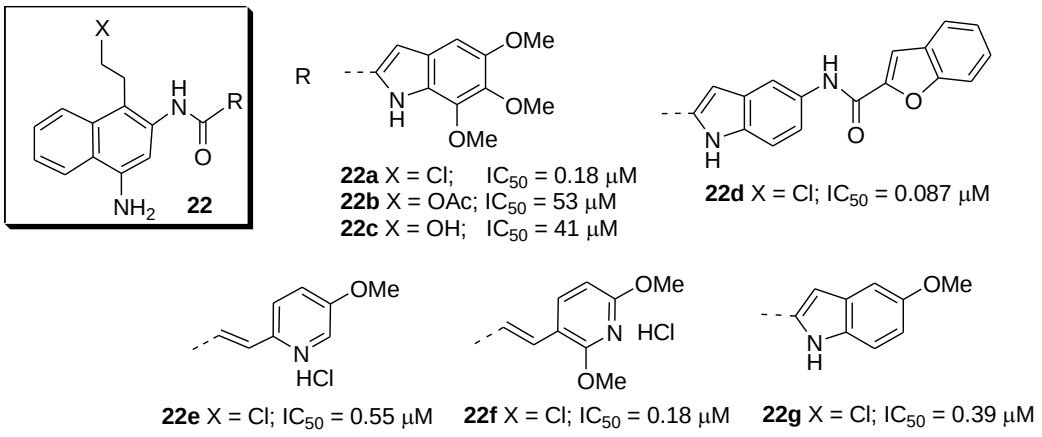
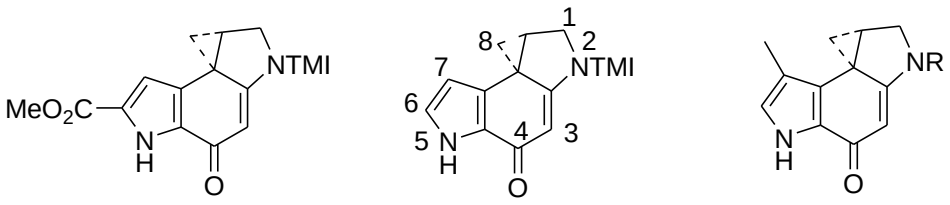


Figure (**11**). Achiral amino-CBI analogues. Cytotoxicity tested against L1210.

1,2,8,8a-tetrahydrocyclopropa[*c*]pyrrolo[3,2-*e*]indol-4-one (CPI)

The CPI subunit, which lacks the C7 methyl substituent of CC-1065 and the C6 methoxycarbonyl group of DSA (Fig. **12**), has been investigated with regard to its alkylation properties using w794 duplex DNA and cytotoxicity against L1210 cells (Fig. **13**). The DNA alkylation selectivity of the CPI pharmacophore was shown to be identical to that of the natural products. A comparison of the relative rate of DNA alkylation of CPI vs. CC-1065 and DSA showed that the presence of the C-6 ester in DSA increased the rate and efficiency of alkylation, whereas the presence of the C-7 methyl group of CC-1065 caused a slight decrease in alkylation rate and efficiency (Fig. **12**). This reflects the effect of each group on non-covalent binding in the DNA minor groove; the ester enhances binding by increasing the overall length of the conjugated system, whereas the steric hindrance of the methyl group decreases binding [49]. Structure activity studies (Fig. **13**) showed that the methoxy substituents of the DSA-TMI group and the extended right-hand chain of CC-1065 are not required for sub-nanomolar activity.



DSA	CPI	C7-Me-CPI (CC-1065 subunit)
IC_{50} = 0.006 nM (L1210)	IC_{50} = 0.007 nM (L1210)	IC_{50} = 0.02 nM (L1210)
Alkylation rate 12-13	Alkylation rate 1	Alkylation rate 0.25
Alkylation efficiency 10	Alkylation efficiency 1	Alkylation efficiency ~0.25

Figure (**12**). Comparison of the biological activity of DSA, CPI and Me-CPI compounds.

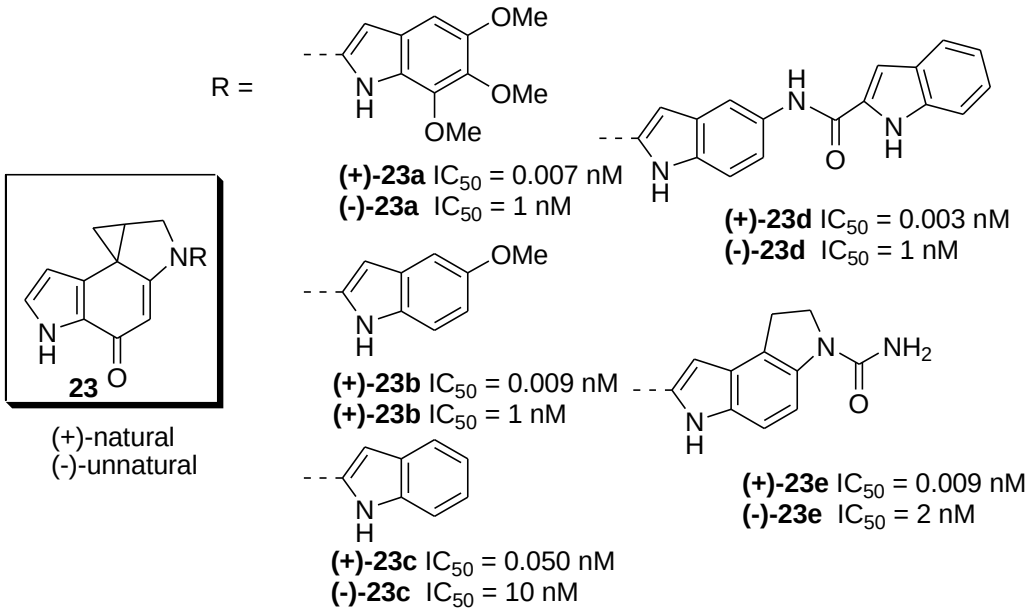
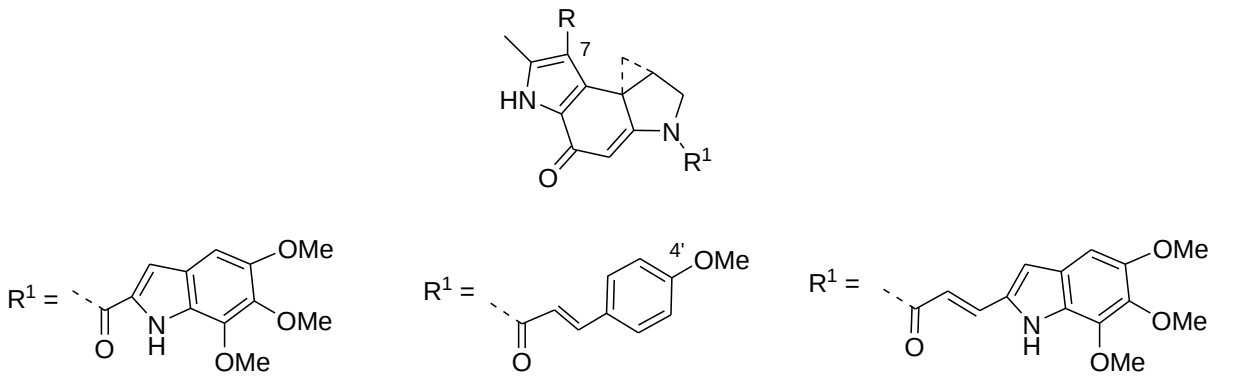


Figure (**13**). CPI analogues. Activity against L1210 cell line

Extensive SAR studies on modification of the CPI subunit have been reported [50-54] including the alteration of the C7 position of the CPI unit bearing either trimethoxy indolecarboxyl (TMI) (**24a**-**g)** [50] methoxycinnamoyl (**25a**-**g**) [50, 53] or acryloylindole (**26**) minor groove binding subunits, (Fig. **14**). Among the C7-substituted CPI-TMI analogues the C7-methyl (**24b**), C7-hydroxymethyl (**24c**), and C7-halogeno (**24d**) derivatives showed potent anti-proliferative activity whereas compounds with electron-withdrawing amide (**24f**) and ester (**24a**, **24g**) groups showed decreased activity. The chloro- (**25d**) and bromo- (**25e**) methoxycinnamoyl compounds possessed remarkably potent antitumour activity (tumour volumes 0.21 and 0.11 of control respectively) with low peripheral blood and bone marrow toxicity (and without causing delayed death). The acryloylindole analogues (**26**) were also highly cytotoxic, and showed potent antitumour activity against murine sarcoma 180 (tumour volume 0.20-0.25 of control) with lower peripheral blood toxicity compared to DSA [52].



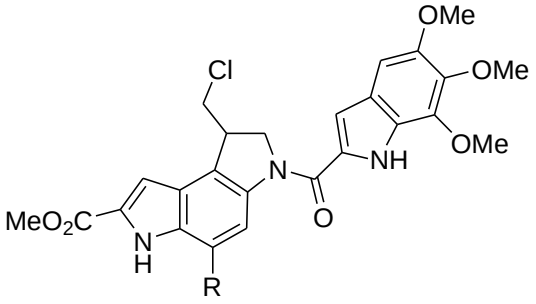
24a R = CO_2Allyl; IC_{50} = 0.43 nM
24b R = Me; IC_{50} = 0.098 nM
24c R = CH_2OH; IC_{50} = 0.093 nM
24d R = Cl; IC_{50} = 0.018 nM
24e R = COMe; IC_{50} = 0.27 nM
24f R = CONHPh; IC_{50} = 16 nM
24g R =CO_2Bn; IC_{50} = 0.74 nM

25a R = CO_2Allyl; IC_{50} = 11 nM
25b R =Me; IC_{50} = 7.5 nM
25c R = CHO; IC_{50} = 0.96 nM
25d R = Cl; IC_{50} = 1.4 nM
25e R = Br IC_{50} = 2.9 nM
25f R = COMe; IC_{50} = 3.3 nM

26a R = H; IC_{50} = 0.49 nM
26b R = CO_2Me; IC_{50} = 0.36 nM
26c R = CHO; IC_{50} = 0.044 nM
26d R = Br; IC_{50} = 0.058 nM

Figure (**14**). Substitution effect on CPI analogues. Activity against HeLa cells, 1 h exposure time

A series of amino-*seco* DSA analogues (**27a-c,** Fig. **15**) yielded agents possessing similar DNA alkylation patterns to the amino-CBI congeners (Fig. **9**), albeit 15-60 fold less cytotoxic against AA8, EMT6 and SKOV3 cell lines (data from the latter reported in Fig. **15**). Methylation of the amine (**27b**, **c**) provided analogues that were slightly more cytotoxic against human cancer cell lines [46].



(±)-27a R = NH_2; IC_{50} = 16 nM
(***S***)-**27a** R = NH_2; IC_{50} = 11 nM
(***R***)-**27a** R = NH_2; IC_{50} = 23 nM
(±)-27b R = NHMe; IC_{50} = 2 nM
(±)-27c R = NMe_2; IC_{50} = 12 nM

Figure (**15**). Amino-*seco*-DSA analogues (IC_{50} values from SKOV3 cell line)

1,2,9,9a-tetrahydrocyclopropa[*c*]pyrido[3,2-*e*]indol-4-one (CPyI)

Comparative trace metal analyses of cancerous and noncancerous human tissues have revealed significant differences that are potentially exploitable from a drug design point of view [55]. One study disclosed the synthesis of the 8-ketoquinoline CPyI structure (Fig. **16**) as a pharmacophore that was expected to provide a tunable means for activation towards alkylation via selective metal cation complexation. The activation of CPyI towards nucleophilic addition by metal cations was found to directly correspond to the established stabilities of the resulting metal complexes ($Cu^{2+} > Ni^{2+} > Zn^{2+} > Mn^{2+} > Mg^{2+}$), providing the opportunity to selectively activate the agents upon addition of the appropriate Lewis acid. The CPyI-based agents (**28a**-**28e,** Fig. **17**) had intrinsic stability comparable to that of CC-1065 and were more reactive than the corresponding DSA and the CBI-based agents [56, 57]. Cytotoxicity is correlated with relative stability, and additionally with the length of the molecule, reflecting its capacity for non-covalent interactions with the DNA minor groove.

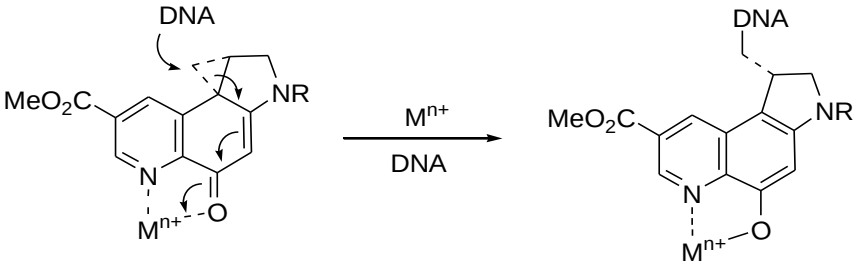


Figure (**16**). Mechanism of metal cation activation towards DNA alkylation

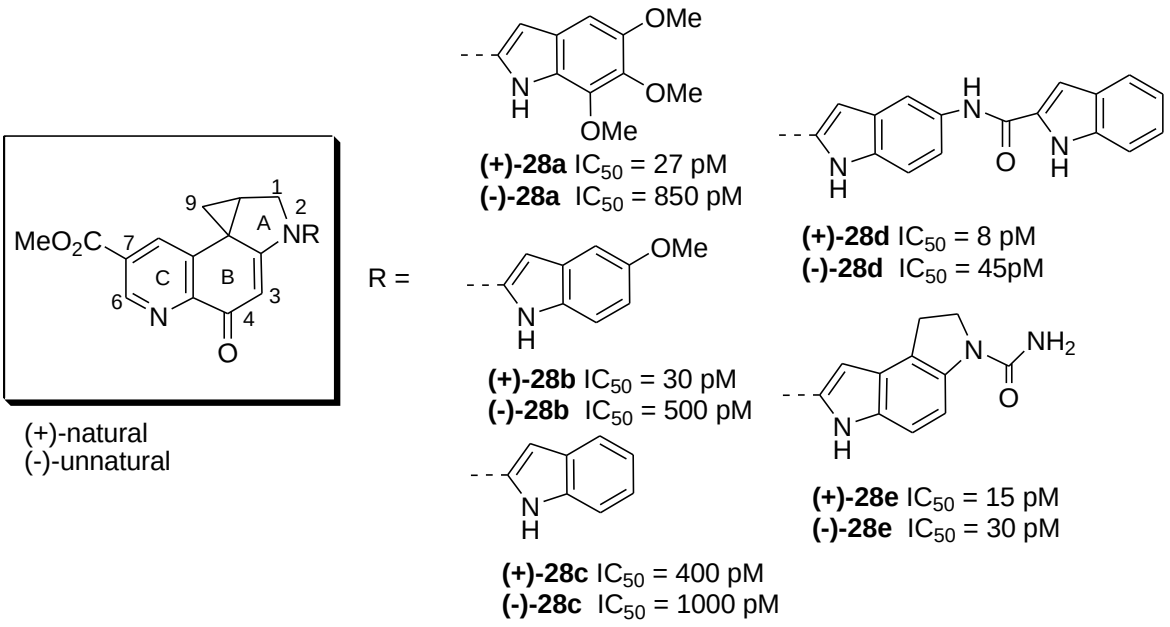


Figure (**17**). *In vitro* cytotoxicity activity of metal-chelating CPyI analogues against L1210

The synthetic route to CPyI molecules lends itself to the preparation of reversed analogues such as **29** (Fig. **18**) [57]. These maintain the DNA alkylation selectivity inherent to all duocarmycin-type molecules, but display a greatly reduced rate of DNA alkylation in the absence of an added metal catalyst. On the addition of a cation such as Zn^{2+}, the rate and efficiency of DNA alkylation increase to equal that of the natural products. *In vivo* studies and potential applications of reversed CPyIs in the treatment of tumours with known differential Zn^{2+} levels have not yet been reported.

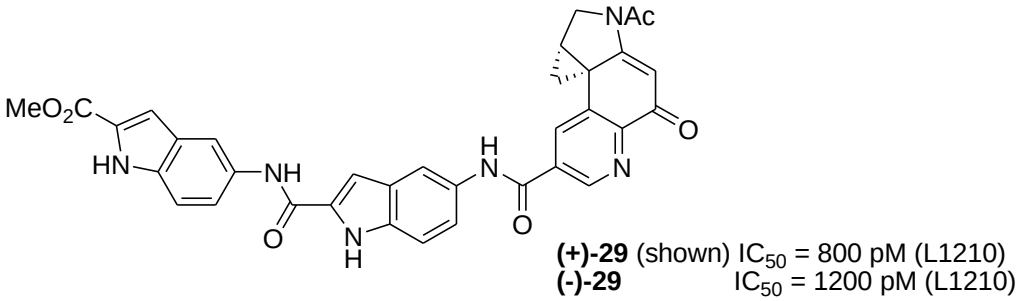


Figure (**18**). Reversed CPyI analogue.

1,2,8,8a-tetrahydrocyclopropa[*c*]pyrazolo[4,3-*e*]indol-4-one (CPzI)

The *N*-Boc-CPzI (Fig. **19**) alkylating subunit provided a further validation of the linear relationship between solvolysis stability, cytotoxic potency and the electron-withdrawing properties. The N-Boc derivatives (**30**) showed modest *in vitro* activity [58]. Two racemic analogues, (**31a**) and (**31b**) (closely related to adozelesin (**66**)) were subsequently synthesized and showed picomolar activity *in vitro*; **31b** was shown to possess moderate *in vivo* activity against L1210 tumours in mice [58-60]. The additional electron-withdrawing nitrogen in the pyrazole nucleus is proposed to play a similar role to that of the carbomethoxy group of the DSA subunit

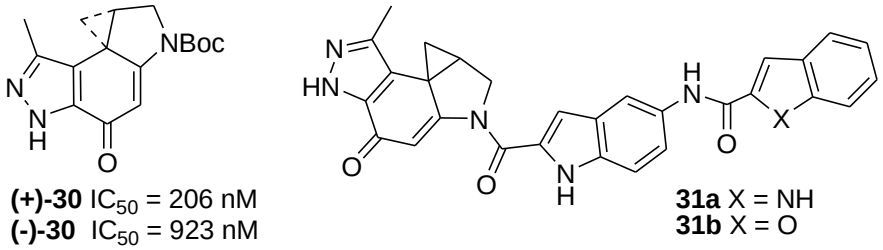


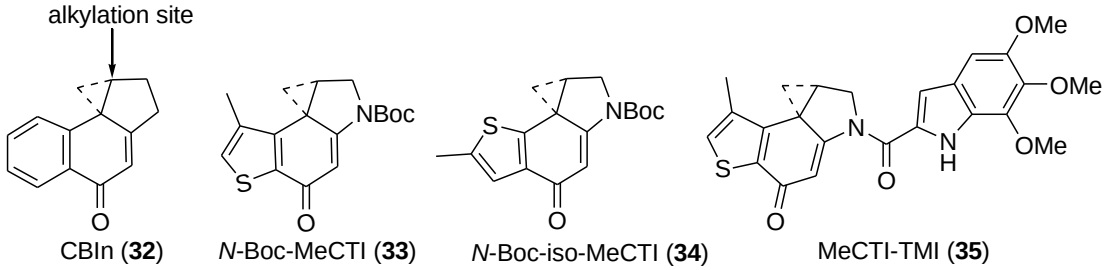
Figure (**19**). Evaluation of the CPzI compounds.

Other heterocyclic DNA mono-alkylating subunits

Interestingly, the examination of the solvolysis and reactivity of a carbocyclic analogue, i.e. 1,2,9,9a-tetrahydro-1*H*-cyclopropa[*c*]benz[*e*]inden-4-one, CBIn (**32**) (Fig. **20**), revealed that removal of the nitrogen and resulting vinylogous amide stabilization increased the reactivity and reversed the inherent regioselectivity of the alkylation reaction, with attack of the nucleophile occurring at the more substituted position of the cyclopropane [33].

Thiophene based alkylating subunits MeCTI, (**33-35**) (Fig. **20**) have also been reported [16]. It was expected that substitution of the heterocyclic nitrogen with sulfur would result in a more stable alkylating subunit than the CPI pharmacophore. Indeed, it was shown

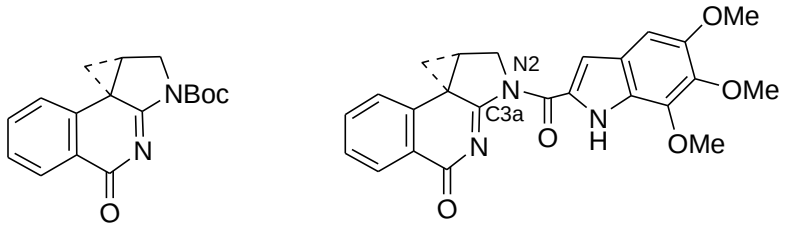
that the acid catalyzed solvolysis of *N*-Boc-MeCTI (**33**) and *N*-Boc-iso-MeCTI (**34**) were similar in reactivity but 5-6 fold more stable ($t_{1/2}$ at pH 3 = 206 and 209 h) than *N*-Boc-CBI ($t_{1/2}$ = 133 h) and *N*-Boc-MeCPI ($t_{1/2}$ = 37 h). Their TMI-derivatives (e.g. MeCTI-TMI, **35**) were found to be equipotent with (+)-DSA (IC_{50} = 0.005 nM and 2 nM for the natural and unnatural enantiomers respectively in L1210).



CBIn (**32**) *N*-Boc-MeCTI (**33**) *N*-Boc-iso-MeCTI (**34**) MeCTI-TMI (**35**)

Figure (**20**). CBIn and thiophene-based pharmacophores.

X-ray analysis of N-Boc-CBA (**36**) (Fig. **21**) showed the molecule to be almost structurally identical with CBI in stereoelectronic alignment of the key cyclopropane, its bond lengths, and the bond length of the diagnostic C3a-N2 bond (which reflects the extent of vinylogous amide conjugation). Despite these structural similarities, the 1,2,9,9a-tetrahydrocyclopropa-[*c*]benz[e]-3-azaindol-4-one (CBA) analogues (**37**) were found to be more prone to solvolysis and hydrolysis, approximately 1000-fold less effective in DNA alkylation, and 100-1000-fold lower in cytotoxic activity than the corresponding CBI derivatives [61, 62].



(+)-36 (shown) IC_{50} = 4000 nM **(+)-37** (shown) IC_{50} = 50 nM
(-)-36 IC_{50} = 9000 nM **(-)-37** IC_{50} = 3000 nM

Figure (**21**). CBA analogues with activity against L1201 cells.

The synthesis and biological investigations of achiral *seco*-6,7-dicarbomethoxy-CPI compounds (Fig. **22**) have been disclosed [63]. The ability of compounds (**38**) and (**39**) to covalently interact with DNA was studied using a thermally-induced DNA cleavage assay. It was found that these agents generally showed similar alkylation sequence selectivity to CC-1065 (**1**) and adozelesin (**66**), which supports the concept that the cyclopropane chiral centre confined by the indoline is not required for potent biological activities [63]. Indeed, these compounds exhibited low nanomolar cytotoxicity against human cancer cells. Further cytotoxicity screening of compound **39** conducted against the NCI panel of 60 human cancer cell lines found it was particularly active against cell lines derived from patients with lung, colon, CNS, skin and breast cancers.

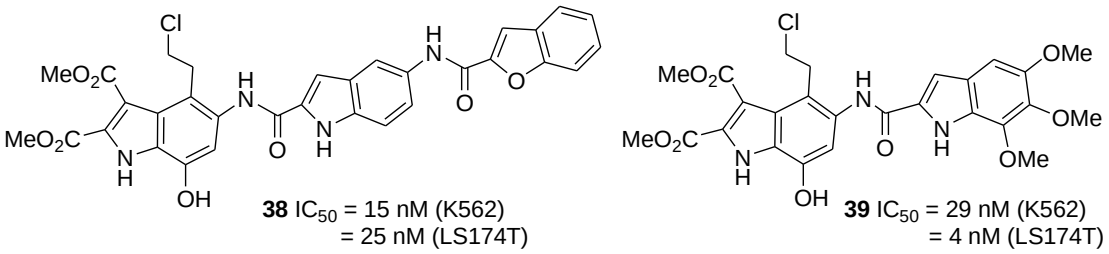
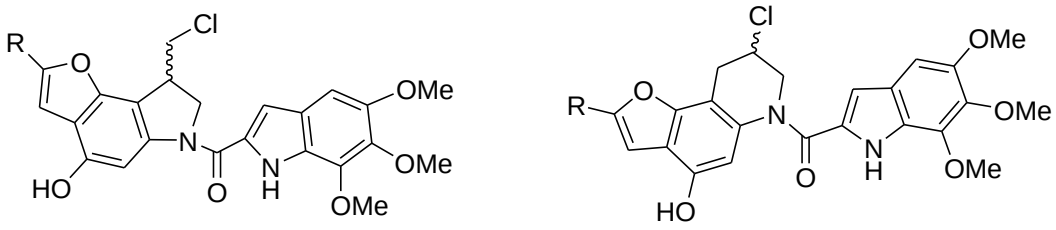


Figure (**22**). Achiral *seco*-analogues of duocarmycin SA

Preparation of *seco*-iso-cyclopropylfurano[2,3-*e*]indoline (*seco*-iso-CFI (**40**)) and *seco*-cyclopropyltetrahydro-furano[2,3-*f*]quinoline (*seco*-CFQ) (**41**) classes of agent have been reported [64]. The iso-CFI moiety was designed to take advantage of the conjugation of the lone pair of electrons of the furan oxygen atom with the cyclopropylindolone moiety (Fig. **23**), providing a second vinylogous conjugation to the pharmacophore and stabilizing the cyclopropylindoline functionality toward nucleophilic attack. The CFQ analogues were derived from the side product arising from an unexpected lack of selectivity in the radical

reaction used to form the chloromethylindoline ring. DNA alkylation studies showed that both *seco*-iso CFI and *seco*-CFQ covalently interact with the N3 position of adenine residues in the minor groove. The cytotoxicity of all the compounds were tested against the NCI panel of cancer cell lines and their inhibitory concentrations were observed to be in the low-to moderate nanomolar range, with particular activity against solid tumours, most notably melanoma and renal carcinoma.



seco-iso-CFI-TMI
40a: R = H; IC_{50} = 53 nM
40b: R = CH_3; IC_{50} = 30 nM

seco-CFQ-TMI
41a: R = H; IC_{50} = 64 nM
41b: R = CH_3; IC_{50} = 32 nM

Figure (**23**). IC_{50} data from the L1210 cell line

Bifunctional alkylating agents

Investigation of DSA has revealed that the natural (+)-enantiomer alkylates adenine N3 within selected AT-rich regions with a binding orientation that extends in a 3' to 5' direction from the site of alkylation, whereas the unnatural (-)-enantiomer similarly alkylates adenine N3, but with a binding orientation that extends in the opposite 5' to 3' direction [65]. A subsequent study evaluated bifunctional alkylating agents (Fig. **24**) against the L1210 cell line and demonstrated that the agents containing the natural enantiomer of the alkylating subunit (**41, 42**) were 10-fold more potent than those with the corresponding unnatural enantiomer (**43, 44**) [66]. The alkylating subunit positioned as in the natural products ('left-hand' subunit) also controls the DNA binding orientation and primary site of DNA alkylation;

agents with the natural enantiomer in this position acting like (+)-DSA (binding 3'- to 5' and alkylating 5'-AAA**A**) and agents with the unnatural enantiomer on the left-hand side acting like (-)-DSA (binding 5'- to 3' and alkylating 5'-A**A**AA) [66].

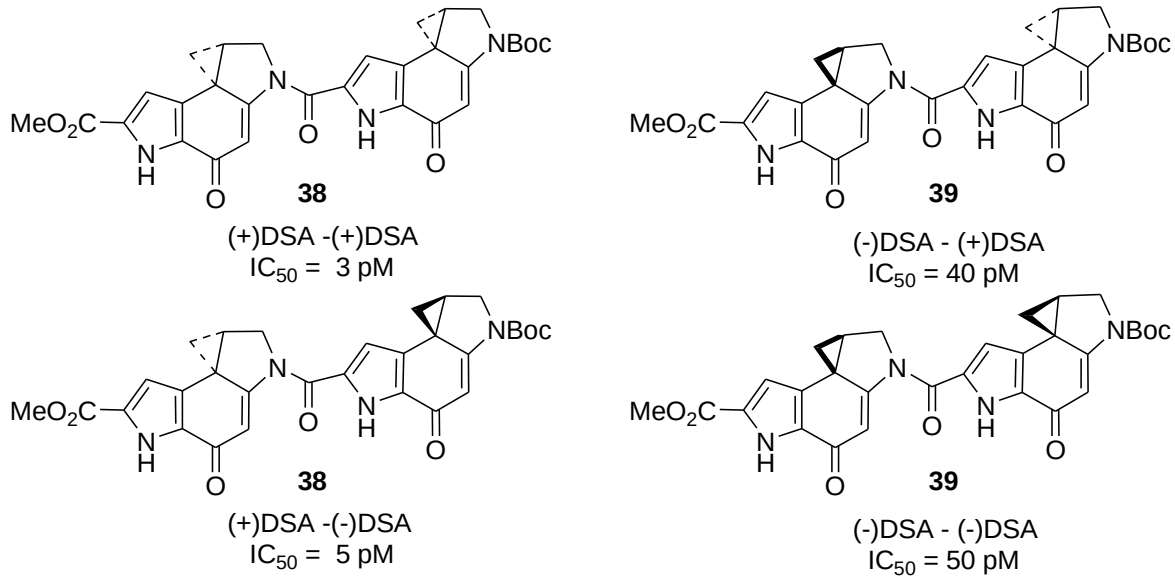


Figure (**24**). Evaluation of bifunctional alkylating agents against L1210 cells.

Racemic *seco*-CBI dimers linked through either C7 or N3 via a flexible methylene chain (Fig. **25**) were tested against the NCI 60 human tumour cell line panel and the order of activity was found to be C7-C7 dimer (**45**) < C7-N3 dimer < N3-N3 (**46**) dimer, with GI_{50} ranging from 41.6 μM to 0.0120 μM [67]. Antitumour activities were also related to linker length and this effect was most noticeable in the C7-N3 series where n = 6 had potencies equal to those of the most active N3-N3 compounds; significant selectivities for selected tumour types also varied with linker length.

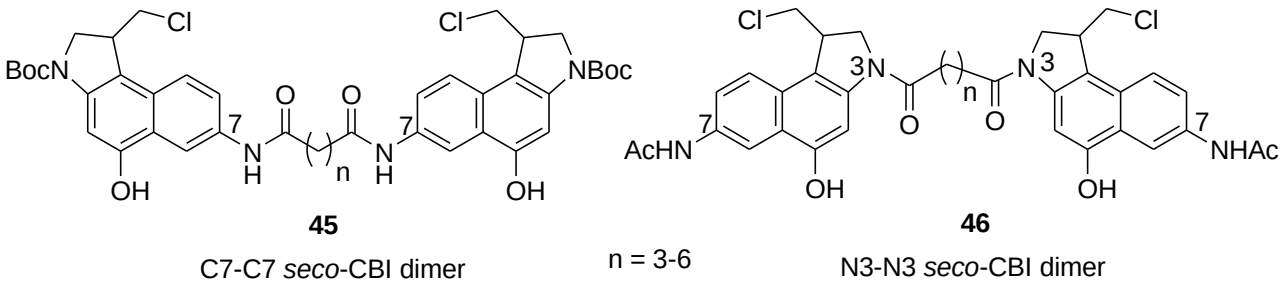


Figure (**25**). *Seco*-CBI dimers

CPI-based bisalkylators with 5,5'-bis(2-carbonyl)-1*H*-indole) (**47**) [69] and 3,3'-(1,4-phenylene)diacryloyl (**48**) [68] as rigid linker groups (Fig. **26**) have demonstrated that an indole-based bisalkylators (**47da**) was more efficient than bizelesin (**68**) *in vivo* (TGI_{50} = 84 μg/kg against colon 26 murine adenocarcinoma). In general the length of the rigid linker had more significant influence on the cytotoxicity and antitumour activity than the type of linker, in contrast to previous work that suggested rigid linkers were superior to flexible ones. The trifluoromethyl-CPI analogue **48a** possessed ultra-potent cytotoxicity (IC_{50} = 0.0027 nM) and showed substantial antitumour activity *in vivo* (TGI_{50} = 0.254 mg/kg) with a therapeutic ratio (MTD/TGI_{50} 30.7) superior to bizelesin (**68**) (MTD/TGI_{50} 3.7) [69]. Bisalkylators (**49**) containing 3,3'-arylenebisacryloyl linkers (Fig **27**) comprising 1 or 2 aromatic rings exhibited potent cytotoxicity and antitumour activity against HeLaS3 human uterine cervix carcinoma cells and colon 26 adenocarcinoma cells respectively with **49a** being the most active *in vitro* and *in vivo* (89% TGI at 1.95 mg/kg against murine Co-26 and 83% TGI at 7.81 mg/kg against human CX-1 colon tumour models) [70].

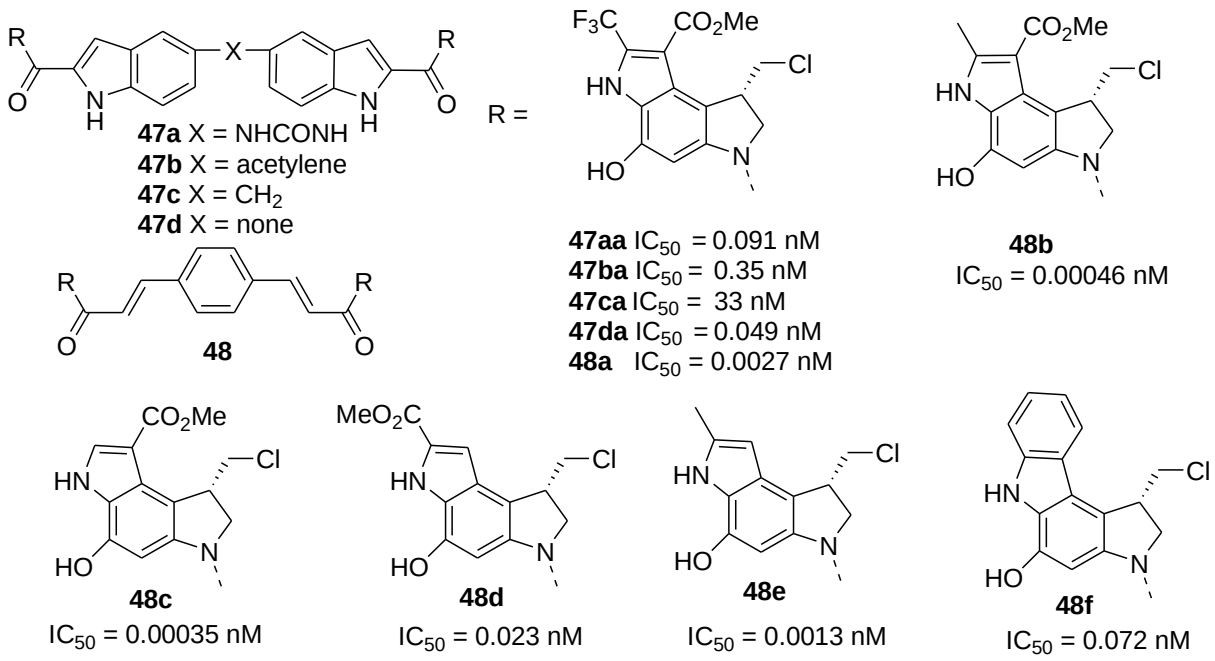


Figure (**26**). CPI bisalkylators containing 3,3'-(1,4-phenylene)diacryloyl linkers. IC_{50} data obtained from HeLaS3 human uterine cervix carcinoma cells.

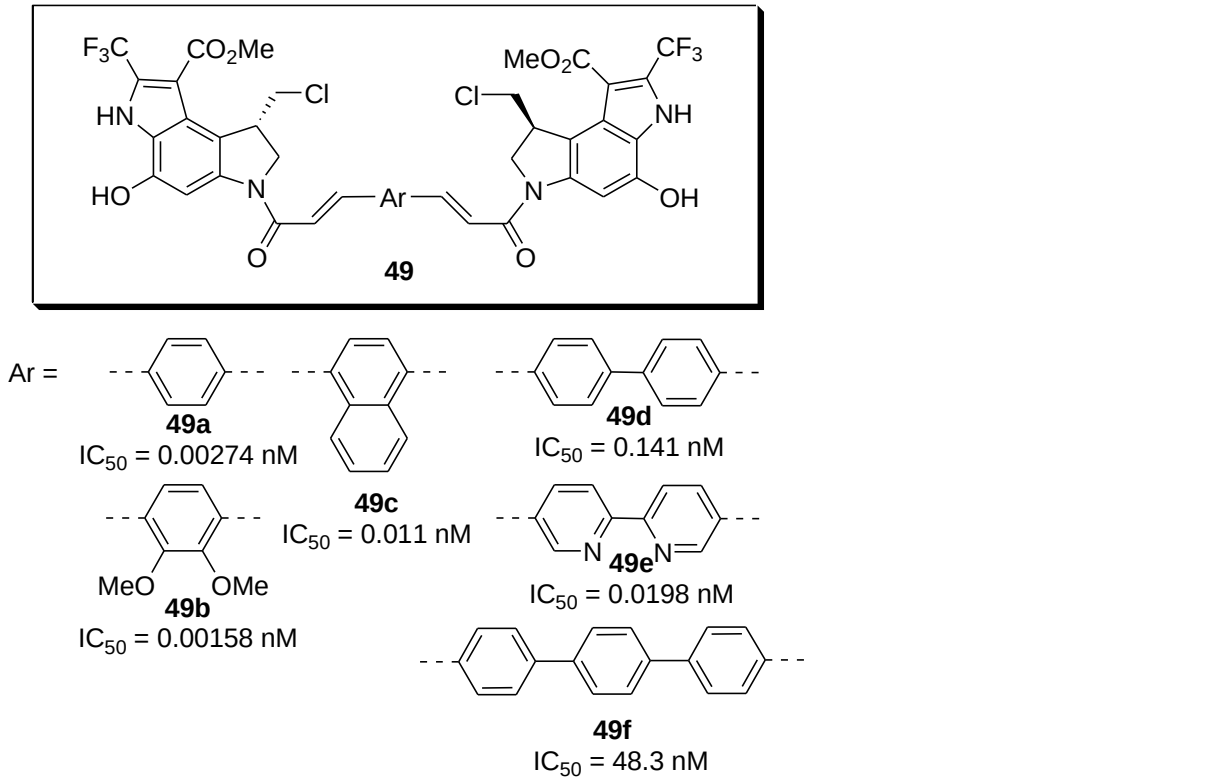
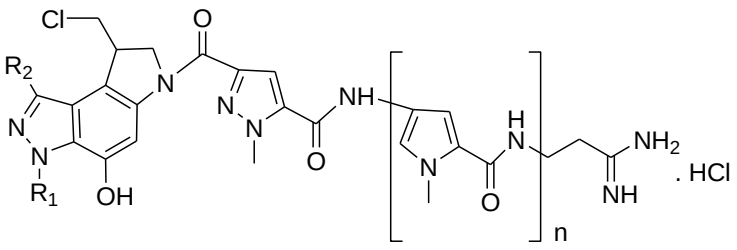


Figure (**27**). CPI bisalkylators containing 3,3'-(arylene)diacryloyl linkers. IC_{50} data obtained from HeLaS3 human uterine cervix carcinoma cells.

Hybrid agents

Hybrids are generally constructs of different molecular entities of both natural and synthetic origin and are synthesized with the hypothesis that combination of structural features of two or more biologically active substances into one molecule may enhance or modulate the therapeutic characteristics of individual components or lead to a completely new properties [71]. In recent years, studies on the combination of duocarmycin-inspired alkylating units with alternative minor-groove binders have yielded hybrid molecules with novel controllable DNA alkylation and cross-linking abilities.

A series of hybrids that combined distamycin A-type polypyrrole minor groove binders with CPzI alkylating units (**50**) (Fig. **28**) have been described. These water-soluble hybrid molecules showed pronounced cytotoxicity against a panel of five tumour cell lines and the hybrid compounds generally showed much greater cytotoxicity than that of the CPzI unit alone (IC_{50} = 520 nM for R^2 = CH_3CPzI). In an investigation of the DNA alkylating properties, the R^1 benzyl analogues were found not to cause DNA alkylation and subsequent strand cleavage. For the molecule to assume a conformation able to alkylate DNA the benzyl group must protrude from the minor groove which is energetically unfavorable. The R^2 methyl series efficiently alkylated DNA in AT-rich regions, with the exact sequence selectivity depending on hybrid length [72].



50a R^1 = H, R^2 = CH_3, n = 1; IC_{50} = 58 nM
50b R^1 = H, R^2 = CH_3, n = 2; IC_{50} = 19 nM
50c R^1 = H, R^2 = CH_3, n = 3; IC_{50} = 7.4 nM
50d R^1 = Bn, R^2 = H, n = 1; IC_{50} = 240 nM
50e R^1 = Bn, R^2 = H, n = 2; IC_{50} = 600 nM
50f R^1 = Bn, R^2 = H, n = 3; IC_{50} = 400 nM

Figure (**28**). CpzI-poylpyrrole hybrids. Activity against L1210 cell line

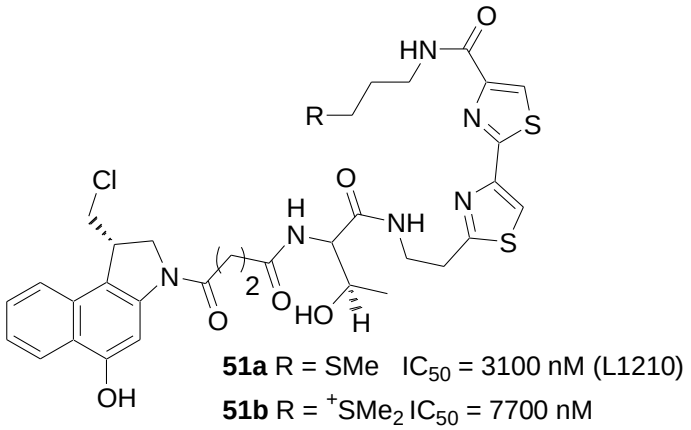


Figure (**29**). Bleomycin-CBI hybrids

Conjugation of the CBI unit with the DNA-binding domain of bleomycin A_2 (a glycopeptide antitumour antibiotic with the ability to cleave DNA) caused a marked reduction in cytotoxicity of the hybrids (**51**) (Fig. **29**) and no change in DNA sequence selectivity; the DNA binding results suggested that the bleomycin domain was acting as an intercalator rather than assisting in delivering the CBI alkylating unit to the minor groove [73].

CPI-lexitropsin hybrids (**52**) (Fig. **30**) containing a *trans*-alkene linker and *n*-propyl end group bind DNA in AT rich regions with slightly different sequence selectivity to CC-1065 and with additional alkylation of guanine [74]. Similar compounds such as **53** have been prepared [75-77]. Hybrid **53** was shown to alkylate double stranded DNA predominantly at the purines of sequence 5'-PyG(A/T)C**Pu**-3' of both strands (sites 1-3) by co-operative homodimer formation, indicating a potential application for duocarmycin hybrids in the development of novel sequence specific DNA-crosslinking agents [75]. Comparison with similar agents where the polyamide is directly bonded to the CPI unit showed that the vinyl linker in **53** increased DNA alkylation efficiency and biological activity (average IC_{50} = 5.62 nM in the Cancer Chemotherapy Centre of Japan's 39 human cancer cell line panel) [77]. Molecular modelling by Lown predicted that a lexitropsin-CBI sandwich hybrid **54** would

have enhanced DNA binding affinity and hopefully enhanced biological potency compared to the CPI hybrid. The results of biological testing on this compound have not been reported [78]. This type of compound is amenable to solid phase synthesis using a linker attached to the CBI amine, thus facilitating library synthesis [79].

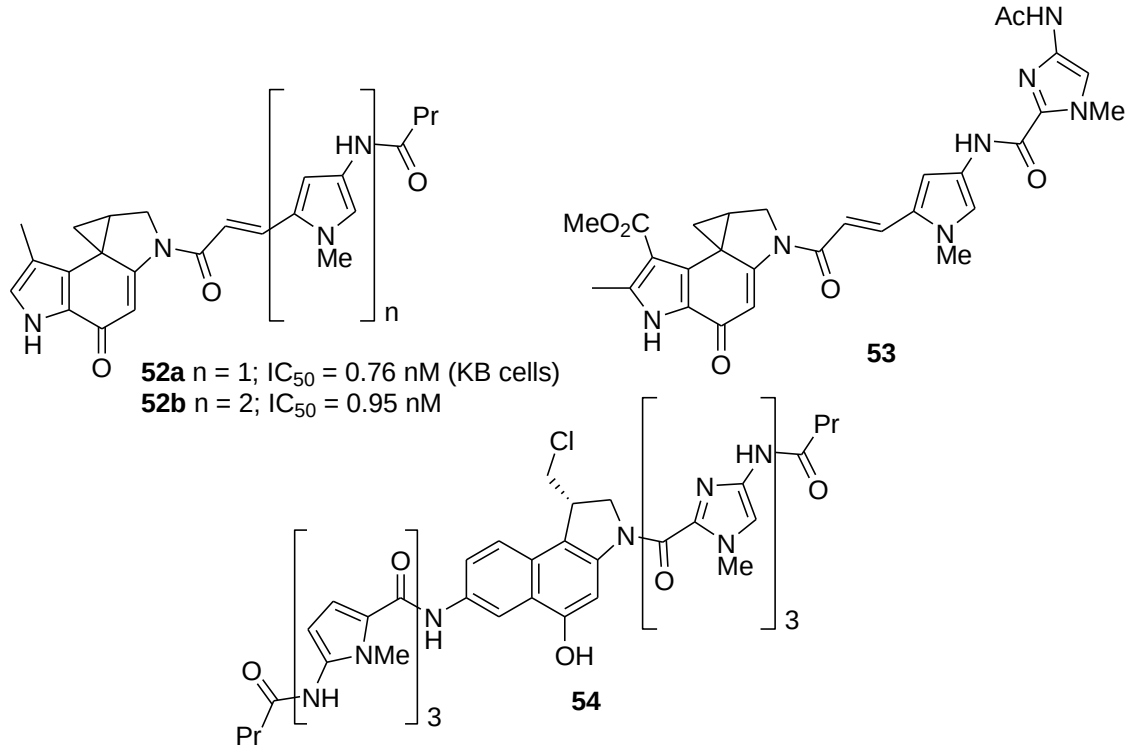
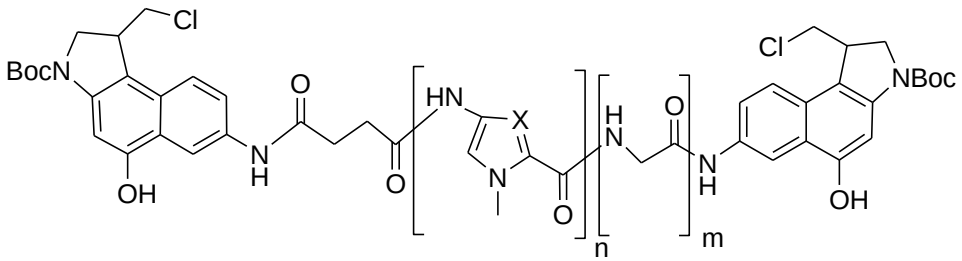


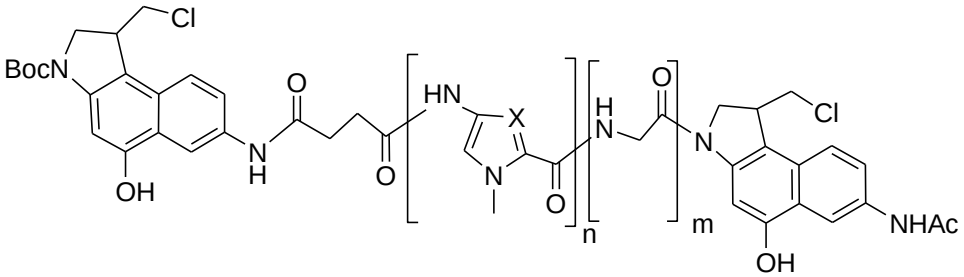
Figure (**30**) Lexitropsin hybrids

Combination of the concepts of hybrid molecules and bifunctional alkylating agents to produce *seco*-CBI-pyrrolo polyamide conjugates **55** and **56** (Fig. **31**) containing two racemic CBI moieties linked to pyrrole polyamides have been reported [37, 80]. Preliminary biological evaluation showed the compounds were active against a panel of 3 human cancer cell lines, but notably less so than the corresponding monomers or alkyl-linked dimers, leading to the hypothesis that overly large molecules may have poor DNA binding affinity, although it has subsequently been shown that very large conjugates can maintain nM activity [81].



X = CH, N, n = 1-3, m = 1-2

55a X = CH, n = 1, m = 1; 91% inhibition
55b X = N, n = 1, m = 2; 87% inhibition



56a X = CH, n = 2, m = 1; 88% inhibition
56b X = N, n = 1, m = 2; 97% inhibition
56c X = N, n = 2, m = 2; 78% inhibition

Figure (**31**). Bifunctional lexitropsin hybrids. Activity: inhibition of growth against the NIH-H460 NSCLC cell line at 1 mM.

The synthesis of CPI and CBI analogues (Fig. **32**) incorporating large imidazole/pyrrole polyamide chains for binding double stranded DNA which can target G-C base pairs has been reported [81-85]. The incorporation of a vinyl linker between the alkylating and minor groove binding agents was again shown to be important for efficient DNA alkylation [75]. DNA alkylation by **57a** has been shown to inhibit gene transcription by alkylation of a site in the gene coding region, suggesting important future applications of these molecules [86]. The natural 12*S* enantiomer of CBI was shown to be an order of magnitude more cytotoxic than the unnatural enantiomer in conjugate **57b** [84]. Both enantiomers of the more complex conjugate **58** showed that the *R* isomer was a faster and more efficient alkylating agents, and that the location of the adenine (site and strand) alkylated could be dependent on the isomer used [87].

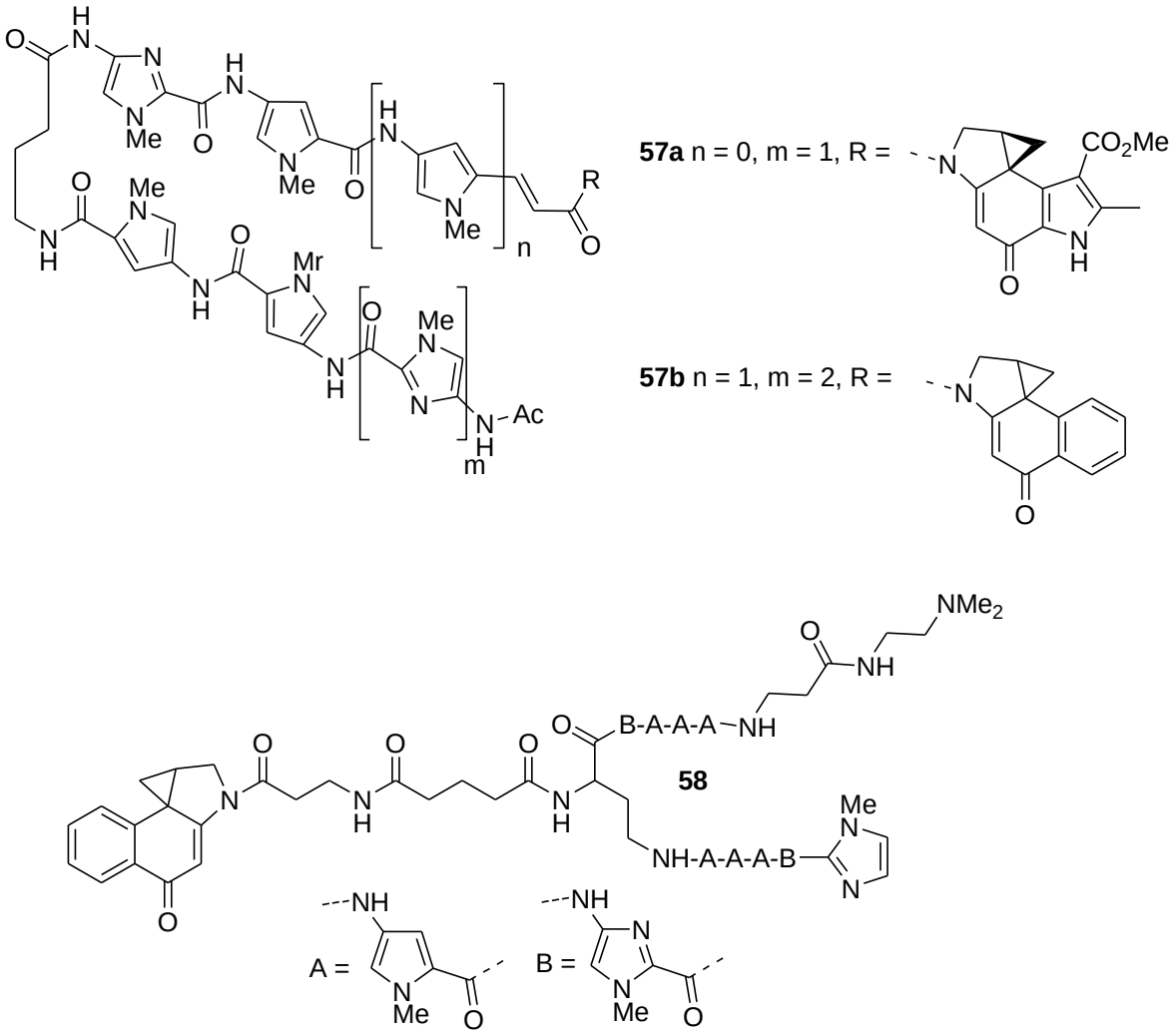


Figure (**32**) CPI and CBI compounds containing imidazole and pyrrole polyamide chains.

Investigation of the DNA alkylation properties of enantiomerically pure lexitropsin-*seco*-CI hybrids (**59**) (Fig. **33**) revealed that the unnatural *R*-enantiomers were more potent DNA-cleaving agents than the natural *S*-enantiomers [88]. Molecular modelling suggested this was due to the closer proximity of the less substituted carbon of the cyclopropane ring (formed *in situ*) to adenine-N3. The achiral CI moiety has been combined with benzofuran and pyrrole/imidazole diamides to produce hybrids such as **60** [89, 90]. These compounds can be targeted to alkylate adenine N3 flanked by a specific DNA sequence by choice of imidazole or pyrrole. These hybrids have reduced cytotoxicity (**60** IC_{50} = 45 μM against L1210 cells) compared to the parent compound.

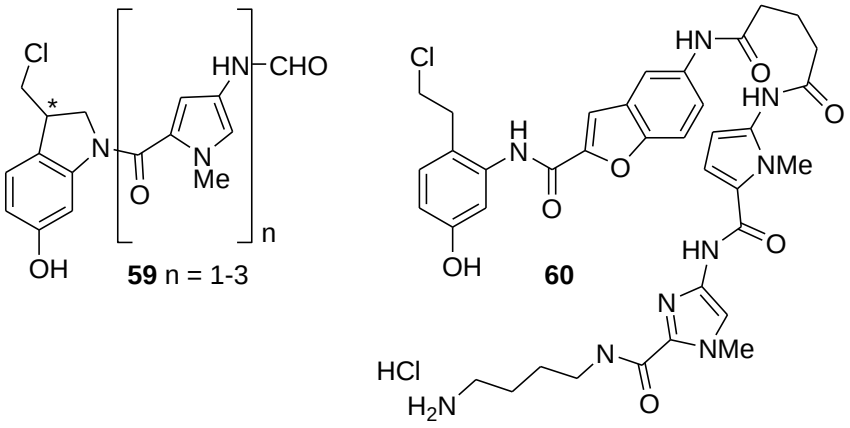


Figure (**33**). Lexitropsin-*seco*-CI hybrids.

A hybrid **61** of the duocarmycin A subunit (produced by hydrolysis of duocarmycin B2) with an imidazole-pyrrole polyamide has been reported (Fig. **34**) [91]. It was shown that the addition of distamycin causes duocarmycins to switch their site of DNA alkylation to N3 of guanine [92], and further that a synthetic pyrrole/imidazole polyamide can be substituted for distamycin to direct the site of DNA alkylation [93]. Hybrids **61** were unreactive with DNA in the absence of distamycin; in its presence they readily alkylated guanine of a nucleotide sequence designed to match the specific hybrid. This technology can potentially be applied to design DNA crosslinking agents with tailored site-selectivity which may have potential applications to gene regulation [94, 95]. For example, combination imidazole-pyrrole-duocarmycin bis-alkylating agents such as **62** have been prepared, which recognizes the nine base-pair sequence 5'-PyGGC(T/A)GCC**Pu**-3', and produce DNA crosslinks only on addition of an activating imidazole-imidazole-pyrrole polyamide.

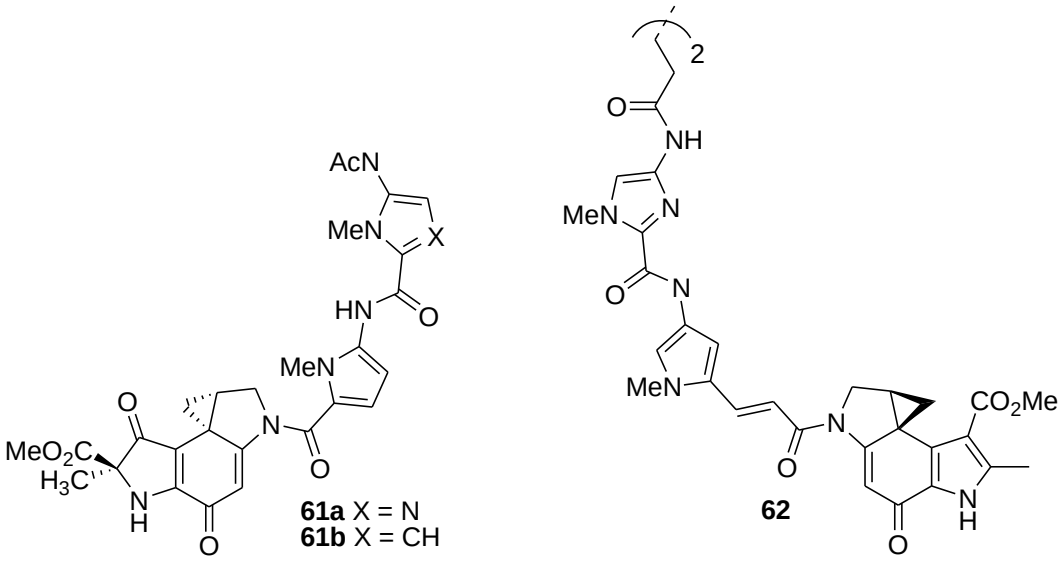


Figure (**34**). Duocarmycin A polyamide conjugate.

Pyrrolobenzodiazepine (PBD) DNA binders have also been examined as hybrids with CI and CBI alkylating subunits (Fig. **35**). PBD analogues bind specifically to 5'-PuGPu-3' sequences, and react covalently to form stable adducts between the imine group of PBD and guanine^2-NH_2 [96]. Since the duocarmycins alkylate DNA on adenine, combining the two agents should create compounds capable of creating interstrand crosslinks between mixed AT and GC sequences over extended segments of DNA, thus creating biological probes with new specificity to interact with novel targets. The prototype CPI-PBD conjugate UTA-6026 (**63**) forms DNA crosslinks across six base-pairs, shows mixed-sequence specific alkylation selectivity, and is highly cytotoxic [97]. More recently, publications on the achiral *seco*-CI-PBD (**64**) demonstrated that such hybrids had enhanced cytotoxicity over CI-TMI (5.6 μM) or amino-CBI-TMI (0.068 μM) alone when measured against P815 murine mastocytoma cells. Both **63** and **64** were found to induce apoptosis in P815 cells. Amino-CBI-PBD (**65**) was shown to covalently react with adenine-N3 positions within the minor groove at AT-rich

sequence, at low concentrations the molecule is selective for a potential interstrand crosslinking site [98].

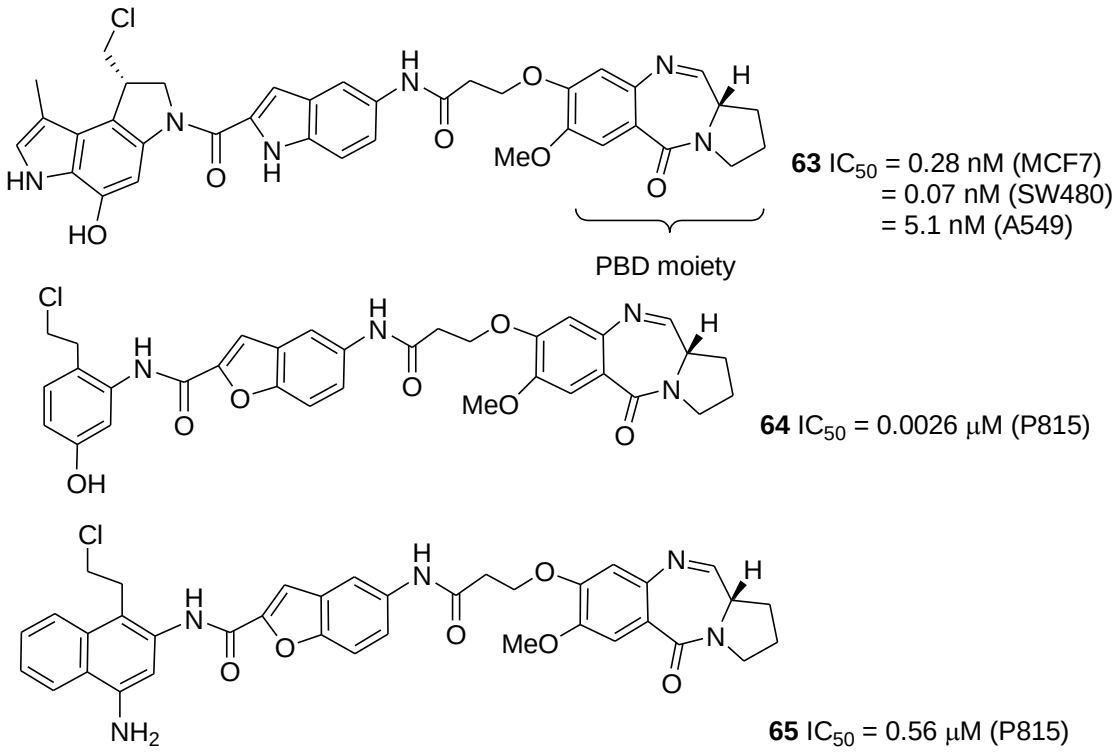


Figure (**35**). Pyrrolobenzodiazepine (PBD) hybrid molecules.

CLINICAL CANDIDATES

Three analogues of CC-1065 (Fig. **36**) with improved therapeutic indices have entered clinical trials. The monofunctional alkylating agents adozelesin (**66**) and carzelesin (**67**) and the bifunctional alkylating agent bizelesin (**68**) have been evaluated in Phase I studies. Although administration of these drugs generally was well tolerated with acceptable toxicities [99-105] only adozelesin (**66**) was further investigated in a Phase II trial. Against patients with metastatic breast cancer, adozelesin (**66**) was shown to have only marginal efficacy with myelosuppression being the most frequent adverse event [106].

KW-2189 (**69**), **a** semi-synthetic derivative of duocarmycin B2 (**4**) [107], was selected for clinical trials due to favorable water solubility (10 mg/mL) and antiproliferative activity in experimental animal models [108-110]. In spite of the deactivating carbamate functionality, KW-2189 (**69**) has been shown to form a covalent adduct with adenine at N3, although it is a 1000-fold more active after cleavage of the carbamoyl moiety by carboxyl esterases *in vivo* [110, 111]. KW-2189 (**69**) has been investigated as a treatment option in patients with advanced malignant melanoma [112] and advanced renal cell carcinoma [113]. In both cases, KW-2189 (**69**) did not show sufficient antitumour activity and a low overall objective response rate was observed with no further studies warranted against these advanced diseases. However, a more recent Phase II trial showed some evidence of antitumour activity in patients with hepatocellular carcinoma and further studies against this disease are warranted [114].

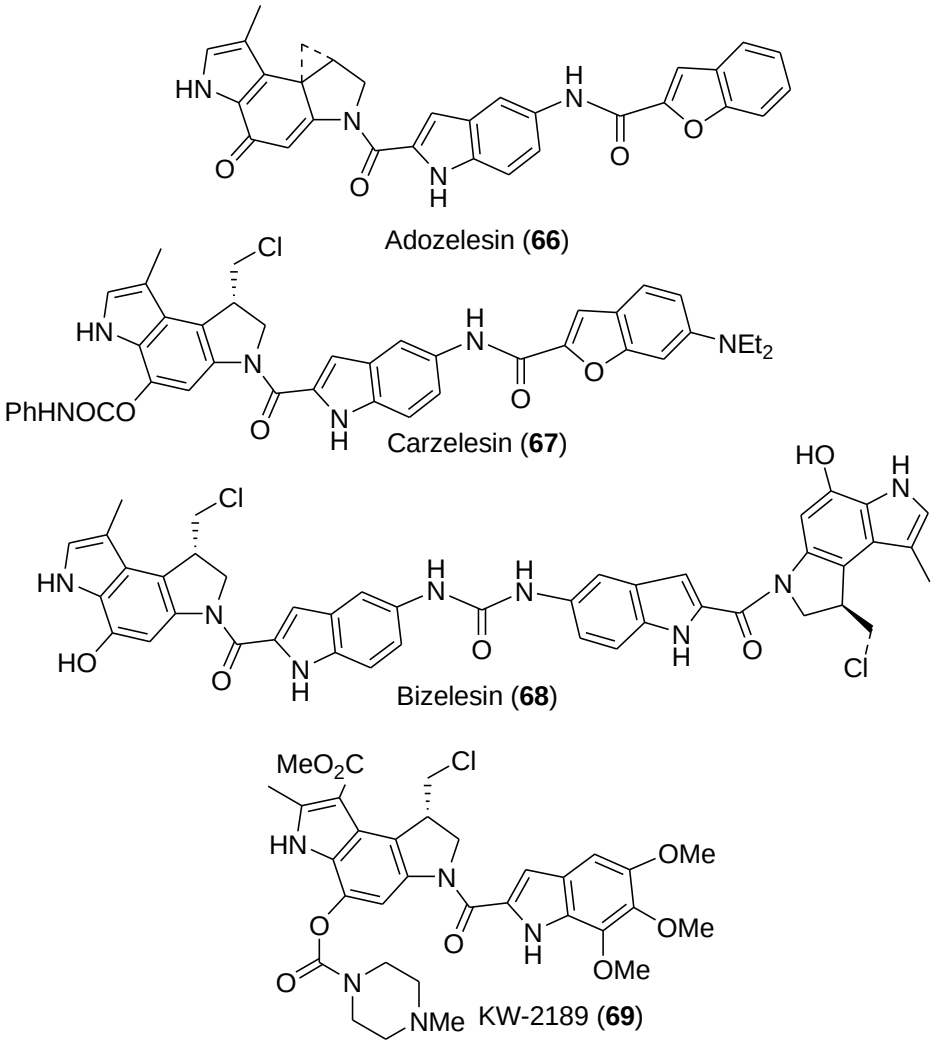


Figure (**36**). Clinical candidates.

PRODRUG DEVELOPMENT

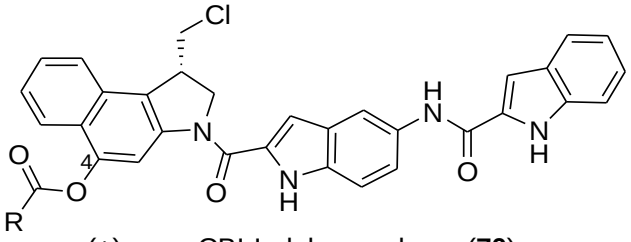
A difficult problem to overcome in cancer treatment is to develop tumour specific chemotherapeutic agents that do not expose normal tissue to the effects of cytotoxic agents such as adozelesin and bizelesin. There is a belief that this problem, along with issues of inability to deliver a curative dose of the agent, unfavorable pharmacological properties such as solubility (a notable problem with duocarmycins), and cross-resistance can be overcome by the development of prodrug forms of cytotoxic agents. The concept of a cancer prodrug requires a tumour-associated enzyme that is able to activate a pharmacologically inactive

compound to the corresponding active parent drug in the vicinity of, or within, the tumour. The generation of potent cytotoxins within the malignant tissue would improve treatment efficacy by allowing higher doses and more frequent treatments, thus minimizing the risks of the occurrence of cross-resistant cancer cells.

A number of different prodrug approaches have been identified, most notably the use of prodrug monotherapy, antibody-directed enzyme prodrug therapy (ADEPT) and gene-directed enzyme prodrug therapy (GDEPT) [115-117]. The success of such strategies depends on the difference in cytotoxicity between the prodrug and the corresponding drug as well as the high biological activity of the drug. Ultrapotent cytotoxic molecules such as the *seco*-duocarmycins are potential candidates in prodrug strategies and this review will in the following sections discuss attempts that have been made to improve on the tumour-selectivity of the CC-1065 and duocarmycin classes of agent.

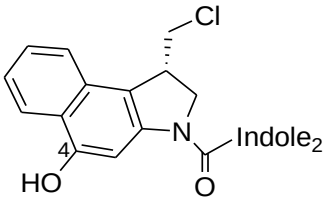
Phenol-based prodrug strategies for cancer monotherapy

It has been demonstrated that duocarmycin and CPI prodrugs formed by acylation of the C4 phenol of appropriate *seco* precursors show improved pharmacokinetics and efficacy. For example, several prodrugs (**70**) of (+)-CBI-Indole$_2$ including carbamates and esters (Fig. **37**) demonstrated picomolar cytotoxicity against L1210 cells, indicating that each prodrug was effectively hydrolyzed to release the free *seco*-drug [34]. Prodrugs **70a** and **70b** are inactive in HCT116 cells (IC_{50} >1600 pM), suggesting that hydrolysis of tertiary amines was not possible in these systems, however **70c-f** showed activity in wild type and drug resistant cell lines (notably **70d**, IC_{50} = 50 pM in HCT116 w/t and a line with reduced topoisomerase II, 100 pM in a MDR HCT116 line).



(+) *seco*-CBI-Indole$_2$ prodrugs (**70**)

hydrolysis

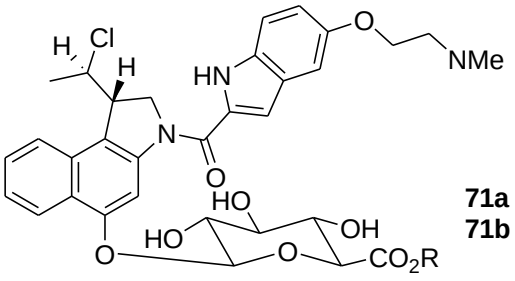


(+) *seco*-CBI-Indole$_2$

(+)-70a R = NMe_2; IC_{50} = 35 pM
(+)-70b R = $N(CH_2CH_2)_2NMe$; IC_{50} = 30 pM
(+)-70c R = NMe_2; IC_{50} = 5 pM
(-)-70c R = NMe_2; IC_{50} =15000 pM
(+)-70d R = NHMe;IC_{50} = 10 pM
(+)-70e R = Me; IC_{50} = 30 pM
(+)-70f R = $CH_2CH_2CON(CH_2CH_2)_2NMe$; IC_{50} = 35 pM

Figure (**37**). Activity against L1210 cells

Glucuronide derivatives of cancer drugs are known to be less toxic, stable in the blood stream, and potentially applicable to the treatment of breast cancer, where the level of β-glucuronidase is higher in human breast cancers than in normal cell [118]. A prodrug of CBI (Fig. **38**) where the phenol is derivatised as the corresponding glucuronide for application as monotherapy in β-glucuronidase overexpressing tumours has been reported [119]. Both the free acid **71a** and ester **71b** (a double prodrug since the methyl ester is cleaved by carboxypeptidases to give **71a**) showed potential as prodrugs with an impressive *in vitro* differential in activity in the presence/absence of β-glucuronidase.



71a R = H; IC_{50} = 610nM/0.9 nM
71b R = Me; IC_{50} = 3300nM/2.1 nM

Figure (**38**) IC_{50}s measured against A549 cell line (absence/presence of β-glucuronidase respectively)

The synthesis and antitumour activity of CBI-bearing ester and carbamate prodrugs (Fig. **39**) has been disclosed. The carbamate prodrugs incorporated methylpiperazine (**72a**), piperidinopiperidine (**72b**), pyridylpiperidine **(72c**) and methylhydrazine (**72d**) whereas the ester prodrugs comprise a hexanoic acid ester (**72f**) and docosahexenoic acid (DHA, **72e**). These prodrugs are expected to be cleaved by carboxylate esterases to release the free cytotoxic metabolite (**73**), in a similar fashion to carzelesin (**67**) and KW-2189 (**69**). The DHA conjugate (**72e**) was of particular interest since DHA is thought to be taken up avidly by tumours from nearby blood supplies, and has been shown to have an inhibiting effect on tumourigenesis [120]; Previously, it had been shown that conjugation of 10-hydroxycamptothecin with DHA greatly improved its efficacy [121]. Ester prodrugs were found to be more potent than amines, and smaller amines more potent than larger ones, indicating their relative resistance to cleavage by carboxylate esterase. *In vivo*, compounds bearing methylpiperazine carbamate (**72a**) and DHA ester (**72e**) moieties were found to possess the highest therapeutic efficacy, without any delayed toxicity. Although **72e** showed higher antitumour efficacy than the corresponding free drug in mice bearing L1210 tumours the effect was less marked than in the 10-HCT conjugate, and there was no significant difference between compound **72e** and free drug **73** in mice bearing Lewis lung carcinoma suggesting that conjugation with DHA cannot be indiscriminately applied to improve a drug's antitumour activity [47, 122].

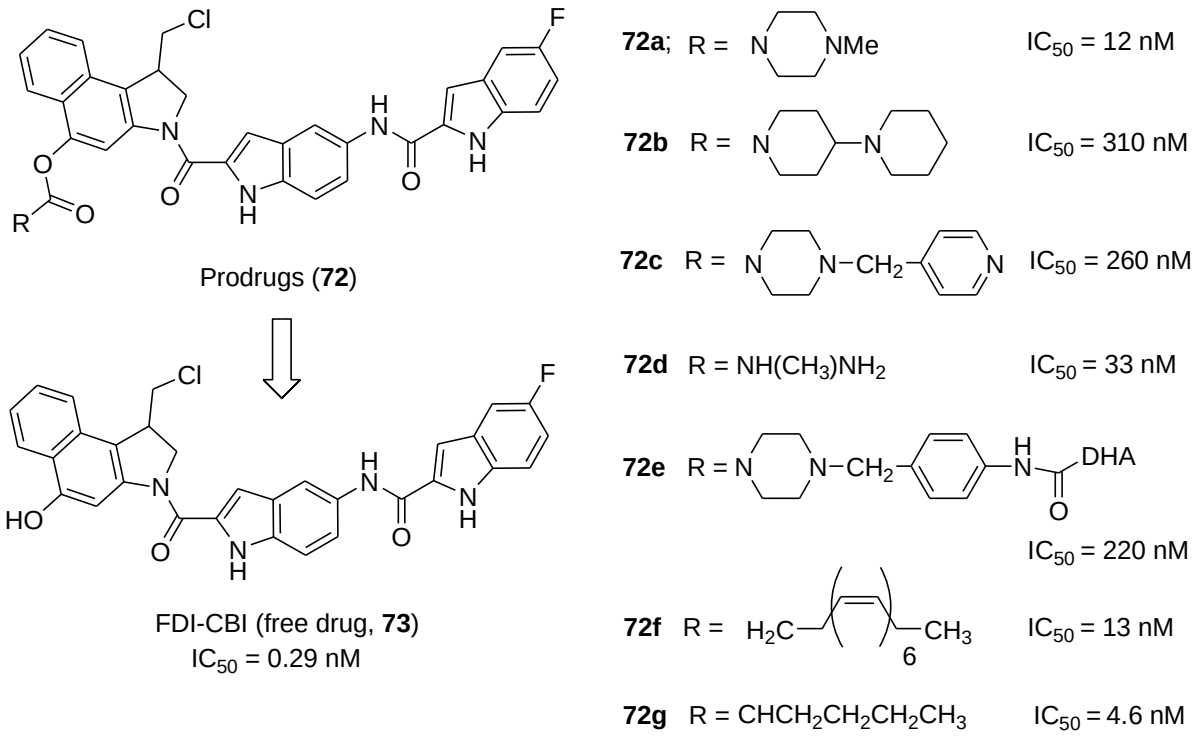


Figure (**39**). Cytotoxicity of racemic compounds against leukemia L1210 cell line.

A series of phenol-based prodrugs with basic C4-*O*-carbamate moieties (Fig. **40**) were developed [54]. It was revealed that while compounds **74a-d** had poor solubility, they possessed good activity *in vitro* and *in vivo* activity. Compounds **74a-d** were shown to possess a varying degree of stability in aqueous solution (1-30h) and enzymatic conversion in mouse and human serum. Some analogues such as **74d** showed remarkably potent antitumour activity *in vivo* (against murine sarcoma 180 and human St-4 xenografts) with low peripheral blood toxicity. Further variants on these compounds included an amine salt on the minor groove binding moiety, which was found to be a better strategy for improving water solubility.

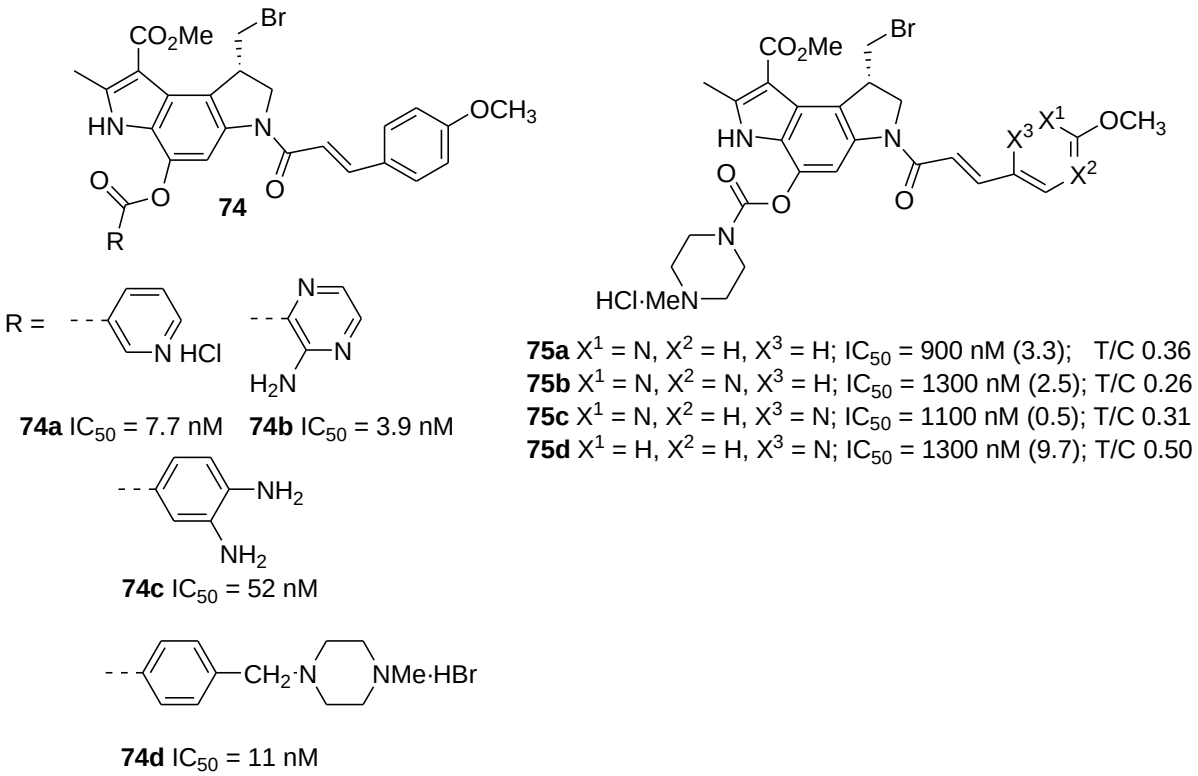
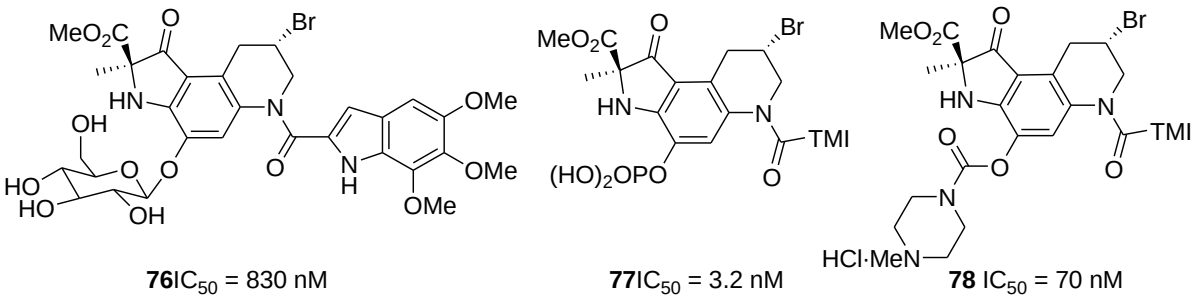


Figure (**40**). *In vitro* activity against HeLa S3 cells (active drug in parentheses for **75**). T/C treated/control tumour volumes in sarcoma 180 xenografts.

Preparation of several other carbamate protected duocarmycin prodrugs bearing 4'-methoxy-β-heteroarylacryloyl groups as the DNA binding subunit (**75**) (Fig. **40**) have been disclosed. Compound **75b** proved to have antitumour activity approaching that of KW2189 *in vivo*. All compounds showed comparably low peripheral blood toxicity and increased water solubility (>20 mM) compared to that compound bearing the trimethoxyindolyl or 4-methoxycinnamate side chains as the DNA binding subunit [123].

Three water-soluble duocarmycin B1 prodrugs (Fig. **41**) were prepared by incorporating a neutral sugar moiety (**76**), a phosphoryl group (**77**) and a *N*-methylpiperazine (**78**) at the free hydroxyl group. Although these derivatives did not alkylate DNA *in vitro*, they exhibited potent *in vitro* cytotoxicity and *in vivo* activity, suggesting that the prodrugs

were activated by enzymatic mechanisms. The carbamate prodrug (**78**) was most efficacious *in vivo* and had superior water-solubility. In further studies against human tumour xenografts resistant to most chemotherapeutic drugs it exhibited potent antitumour activity comparable to that of KW-2189 but without this compound's peripheral blood toxicity [124].



76 IC_{50} = 830 nM **77** IC_{50} = 3.2 nM **78** IC_{50} = 70 nM

Figure (**41**). Water-soluble duocarmycin B1 prodrugs. IC_{50} data obtained from HeLa S3 cells.

Bioreductive prodrugs

Hypoxia generated as a result of a poor and inefficient neovasculature is a characteristic feature of many solid tumours and is associated with the development of an aggressive phenotype and resistance to radiotherapy and chemotherapy. The growth of solid tumours *in vivo* beyond 1-2 mm in diameter is associated with the genesis of new blood vessels [125]. Even though there is progressive formation of neovasculature, there are areas within the tumour that are located at some distance from the blood supply (approx. 50 - 250 μm distance from blood vessels), where the oxygen and nutrient supply is poorly delivered [126]. Hypoxia occurs in well-differentiated, slow-growing, non-metastatic tumours as well as in rapidly growing, anaplastic, aggressive malignancies. Resistance to chemotherapy and radiotherapy is commonly associated with low levels of oxygen in human solid tumours [127]. The difference in normal tissue and hypoxic tissue can be exploited by designing drugs that are cytotoxic only to cells with a very low oxygen level.

Recently *N*-acyl *O*-amino phenol prodrugs of CBI-TMI and CBI-indole$_2$ such as **79** were reported. These compounds were explored for enzymatic reductive activation by cleavage of a weak N-O bond by reducing nucleophiles which are thought to be located in the hypoxic fractions of tumour cells (Fig. **42**). In spite of the NHBoc masking group, the most promising compound was equipotent with that of the free *seco*-drug (IC_{50} = 0.03 nM against L1210) indicating its successful release under the assay conditions [128], although the investigators have not indicated whether this is due to chemical instability or enzymatic metabolism. Regardless of mechanism of activation, **79** showed superior *in vivo* activity to **79a**, with improved efficacy and decreased toxicity (in one study 5/6 mice treated with **79** survived 52 weeks, whereas all those treated with **79a** succumbed to drug-related complications before this stage).

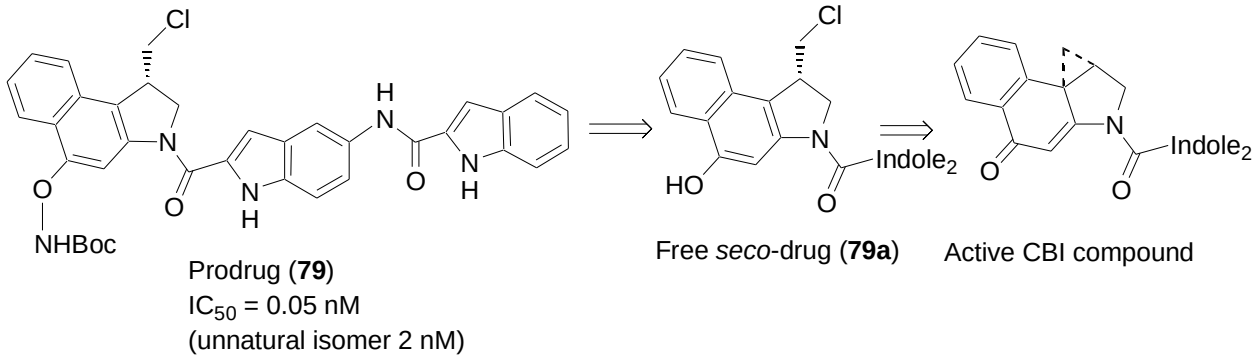


Figure (**42**). *N*-acyl *O*-amino phenol prodrugs of CBI.

A further class of duocarmycin based DNA alkylating agents **81** incorporate the quinone moiety of mitomycin A (**80**) (Fig. **43**), which is thought to impart tumour cell selectivity as a result of preferential reduction and activation in hypoxic tumours. It has been shown that under nonreductive conditions, this quinone analogue did not alkylate DNA even at 10^{-2} M concentration, as the oxidized form cannot undergo spirocyclisation to form the

reactive cyclopropane. The reduced form, hydroquinone **82**, alkylates DNA in a sequence-specific manner identical to the alkylation pattern of the natural product but does so with lower efficiency (100-fold less efficient than duocarmycin SA). A cell-based assay established that these analogues, like the mitomycins, were more active in DT-Diaphorase-enriched cell line (H460) versus a DT-Diaphorase-deficient cell line (H596). Further studies confirmed that they are good substrates for human recombinant DT-Diaphorase, establishing the viable potential for these and related agents such as **83** to be tumour-selective DNA-alkylating agents subject to bioreductive alkylation [129].

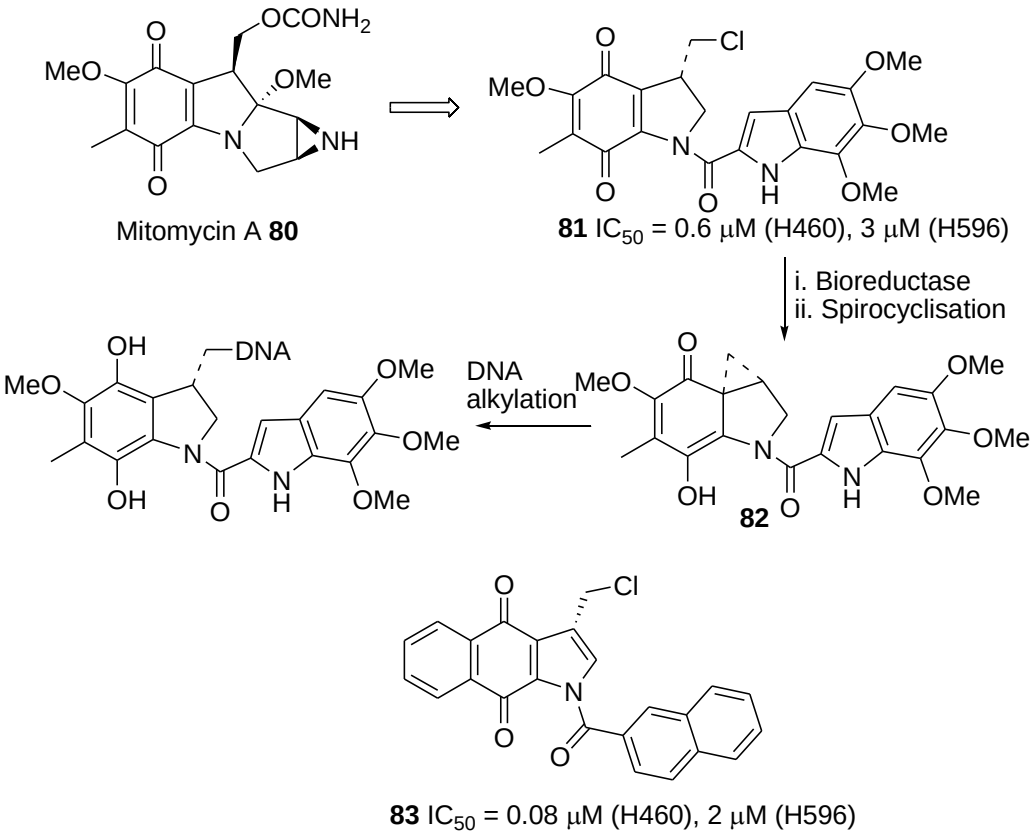


Figure (**43**). Mitomycin A-based compounds.

GDEPT strategy based prodrugs

GDEPT is a two-step treatment for patients with solid tumours. Firstly, a gene expressing the foreign enzyme is delivered and expressed in the tumour. Secondly, a prodrug is administered and converted to a potent cytotoxin by this enzyme [130]. Investigations on this concept have led to the use of oxygen-insensitive flavin mononucleotide nitroreductase (NTR) as potential enzyme for GDEPT application [131]. The potential cytotoxic metabolites, the *seco*-amino chloromethylindoline-containing molecules, have already been described in this review (Fig. **9**). A wide variety of nitro-containing aromatic carbamate prodrugs including 4-nitrobenzene, 2- and 5-nitroimidazole, nitrothiophene, nitrofuran and other substituted carbamates **84a**-**g** (Fig. **44**) have been investigated for their use in the NTR mediated GDEPT therapy [132-134]. In Fig. **44**, selected prodrugs are presented with their cytotoxicity values from the resistant SKOV3 cell line (new compounds were also tested against WiDr, V79 and EMT6 cell lines in the absence/presence of NTR). Although good differential activity was observed *in vitro*, no significant antitumour activity was observed for **84a** [134], **84d** and **84e** [132].

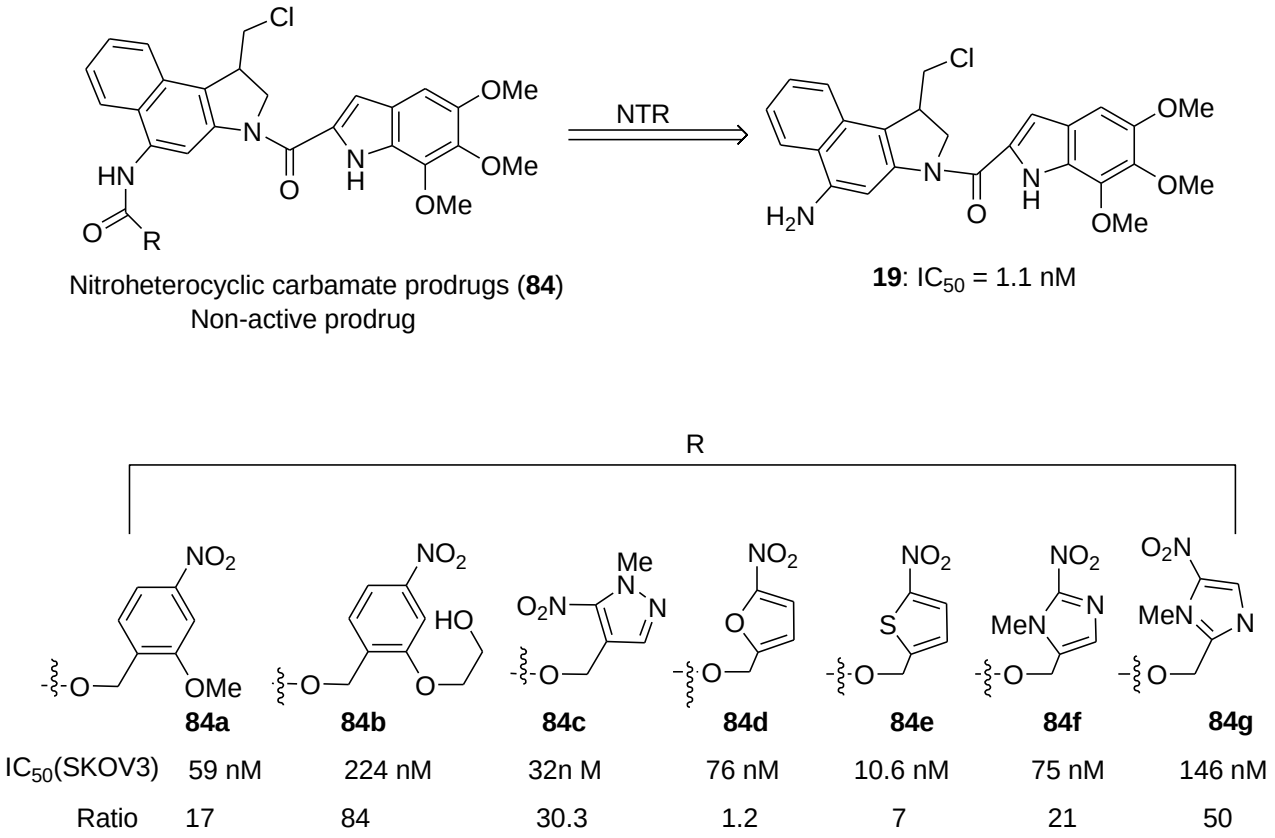


Figure (**44**). Nitroaryl-carbamate CBI prodrugs as candidates for GDEPT. Ratio = Cytotoxicity in the absence and presence of NTR (SKOV3/SKOV3+NTR)

ADEPT strategy based prodrugs

ADEPT is an attractive approach to overcome the problem of insufficient differentiation of normal and malignant cells. As with GDEPT, ADEPT is a two step treatment; after administration of an antibody-enzyme conjugate which binds to an antigen preferentially expressed on the surface of tumour cells, a non-toxic prodrug is administered that is inactive until converted by the tumour cell surface-located enzyme. Ultrapotent compounds such as the *seco*-duocarmycins are potential candidates for [135-142]. The synthesis of the glycoside derivatives of *seco*-CI-TMI (**85**) (Fig. **45**) and the *in vitro* evaluation of its cytotoxic effect has been reported [135]. In spite of its simple structure, as discussed earlier, *seco*-CI-TMI shows a remarkable high cytotoxicity. Exposure of these

glycoside prodrugs to cultured carcinoma A549 cells, in the absence and presence of glucohydrolase led to a significant differential activity, hence showing the potential for ADEPT [135].

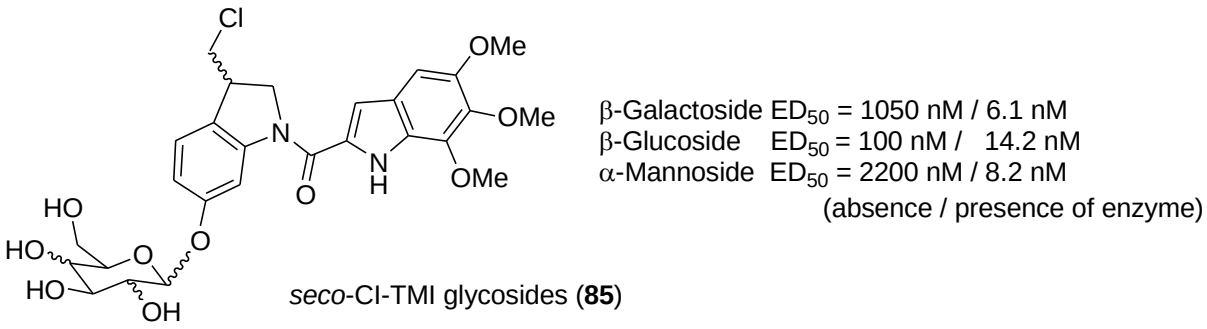


Figure (**45**). Carbohydrate CI prodrugs as candidates for ADEPT.

The design and synthesis of *seco*-CBQ galactoside prodrugs such as **86** (Fig. **46**) containing a secondary chloride that should diminish direct alkylation (which was postulated as a reason for unexpectedly high cytotoxicity in a CBI prodrug with a primary chloride leaving group [143]) through steric hindrance has been reported [144]. Indeed, it was shown that, in the presence of *β*-D-galactosidase, **86** was very cytotoxic against human bronchial carcinoma (A549) and pancreatic ductal adenocarcinoma (PancTu 1) cell lines (IC_{50} = 0.2 nM and 0.13 nM respectively). In contrast, in the absence of the enzyme, the toxicity was decreased by 1600-3140 times in the same cell lines [136].

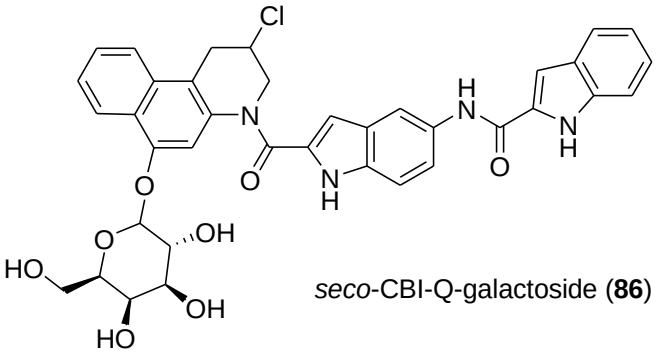


Figure (**46**). *seco*-CBQ galactoside prodrug.

Further galactosylated prodrugs e.g. **87** were designed to reduce cytotoxicity prior to tumour-specific metabolism by incorporating the chloroethyl *seco* group (Fig. **47**). *In vitro* evaluation of two isomers was carried out against human bronchial carcinoma A549 cells in absence and in the presence of *β*-D-galactosidase. Interestingly, the two diasteroisomers shows considerable difference in their activity and it was found that the *anti*-isomer (IC_{50} = 1.3 nM) was more than 200-fold active than the *syn*-isomer (IC_{50} = 280 nM) in presence of the enzyme [136, 143].

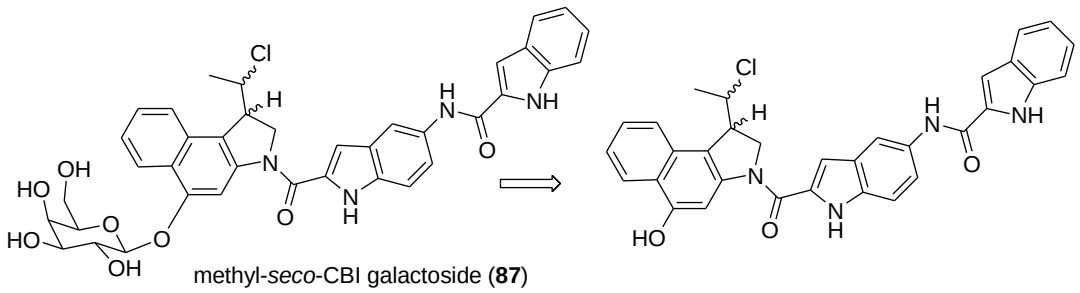
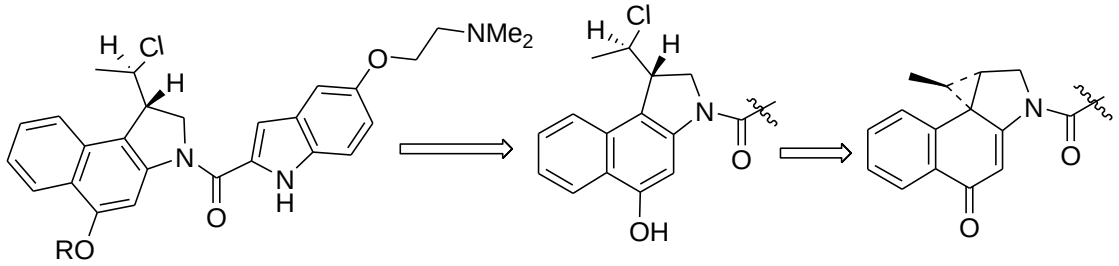


Figure (**47**). Methyl *seco*-CBI galactoside prodrug.

Recent publications have described further prodrugs **88** (Fig. **48**) where the phenolic hydroxyl group is protected as glycoside. The *N*,*N*-dimethylaminoethoxyindole carboxylic acid component facilitates binding in the DNA minor groove and increases water solubility [137, 145]. When tested against the A549 cell line, the galactoside **88a** exhibited micro molar activity, but significantly was shown to be more than 5000-fold active in the presence of *β*-D-galactosidase (IC_{50} = 0.75 nM). In contrast, the (-)-diasteromeric *seco*-agent showed activity 1000-fold lower than that of (+)-isomer [138, 145, 146]. Investigation of the role of the sugar moiety found that the identity of this group was important for prodrug stability; after 24 h

exposure to culture medium, the prototype galactoside **88a** was 72% hydrolyzed, whereas **88b-e** were 94-98% intact [141].



(**87a**) R = D-galactose: IC_{50} = 3600 / 0.8 nM
(**87b**) R = D-glucose: IC_{50} = 2000 / 1.9 nM
(**87c**) R = D-lactose: IC_{50} = 2700 / 2.1 nM
(**87d**) R = D-cellobiose: IC_{50} = 2400 / 2.6 nM
(**87e**) R = D-mannose: IC_{50} = 2700 / 0.6 nM
(**87f**) R = L-rhamnose: IC_{50} = 3500 / ND nM
(**87g**) R = D-glucuronic acid methyl ester: IC_{50} = 3300 / 2.1 nM

(+)-*seco*-CBI
IC_{50} = 0.75 nM

(+) Active drug

Figure (**48**). IC_{50} data obtained from A549 cell line in absence/presence of relevant glycosidase.

Compound **89**, which consists of a conjugate of a *seco*-analogue of CBI and cephalosporin (Fig. **49**) was designed to be activated by β-lactamases. However, the success of this ADEPT approach was limited due the capacity of **88** to readily form the spirocyclopropanehexadienone pharmacophore in non-malignant tissue [147]. In a similar vein, glucuronide derivatives were also prepared, for example of CBI analogues (**90**), which showed a slight decrease in toxicity compared to the de-glucuronidated compound (IC_{50} = 0.6 nM vs. 0.1 nM against U937 leukemia cells) leading the authors to conclude such agents would be more applicable as conventional cytotoxics rather than prodrugs [148].

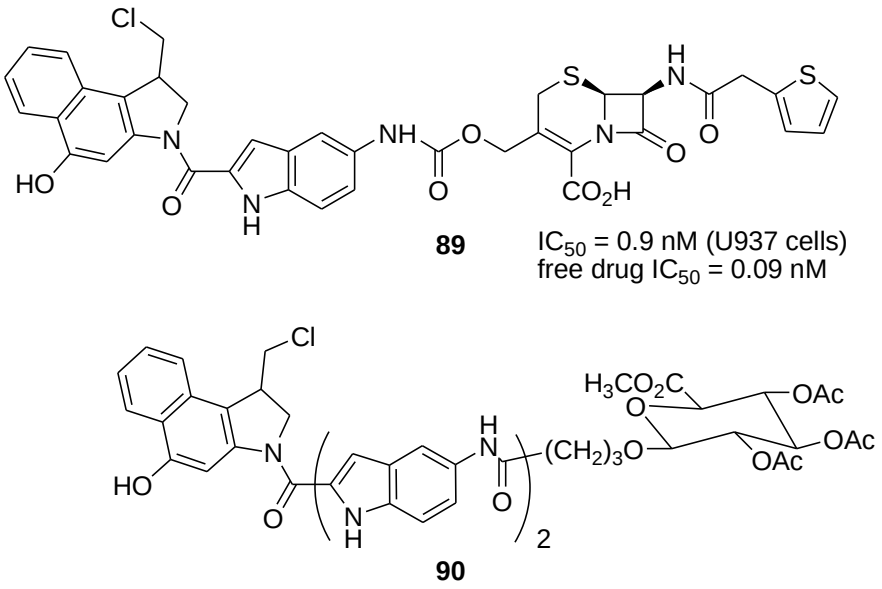


Figure (**49**). Prodrugs of the *seco*-CBI pharmacophore.

Further drug-conjugate strategies

There has been a recent resurgence of interest in the use of covalently-linked antibodies to deliver highly cytotoxic drugs to tumours [149]. Due to their picomolar activity, derivatives of duocarmycins are well-suited for this application. The CBI-based analogue **91** (Fig. **50**) was conjugated to antibodies anti-B4 (against CD19) and N901 (against CD56). The anti-B4 conjugate was highly potent (IC_{50} ~0.1 nM) and completely cured mice bearing large Namalwa B-cell lymphoma tumours [150].

Duocarmycins have also been delivered to tumour cells by a polyethyleneglycol (PEG)-dipeptide linker [151, 152]. The derivative DU-257 **92** (Fig. 50) was attached via a PEG-dipeptide linker to monoclonal antibody KM231, which specifically recognizes sialyl Lewis a (sLe^a) carbohydrate antigen. The conjugate was stable in aqueous solution, and was shown to be specifically activated by SW1116 colorectal adenocarcinoma cells, but a low level of drug release and availability of material precluded a full investigation of the conjugate's efficacy [152].

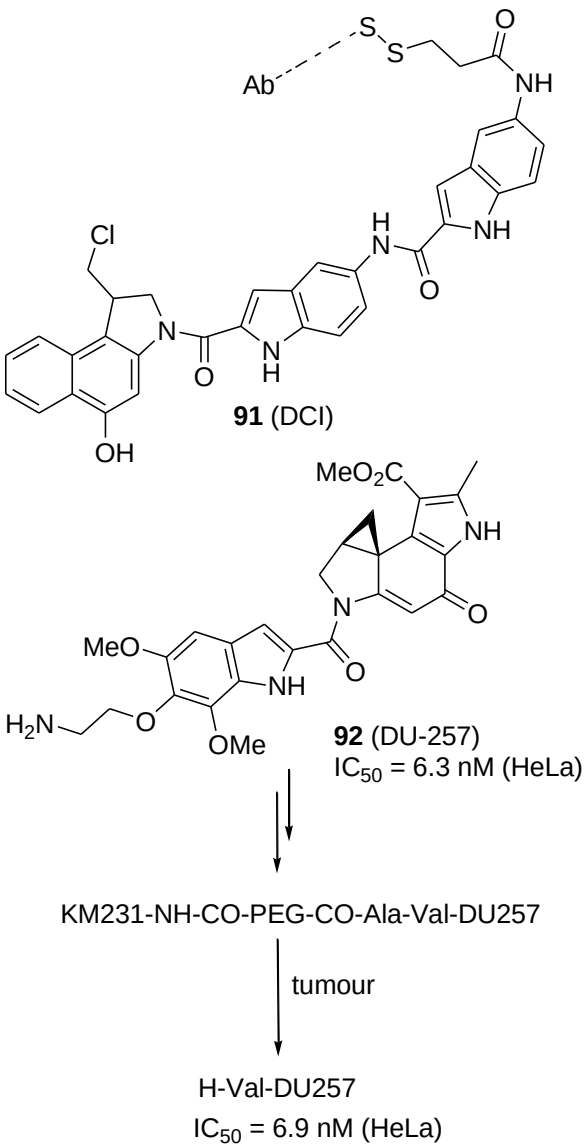


Figure (**50**). CBI and DU-257 antibody conjugates.

Preparation of *seco*-CPyI and *seco*-amino-CBI derivatives conjugated with enzymes through a linker on the phenol or amino moiety respectively have been reported (Fig. **51**) [153]. **93** and **94** were suitable for conjugation with enzymes without causing aggregation (which arises from poor drug solubility). Conjugation with an anti-CD30 antibody reduced cell growth to < 25% of control. The conjugate of **93** with antibody cBR96 was more

cytotoxic than doxorubicin or cisplatin to NCI-H69 cells with high levels of P-glycoprotein (IC_{50} = 13.4 nM) and multi-drug resistant NCI-H69/LX4 cells (IC_{50} = 20.7 nM).

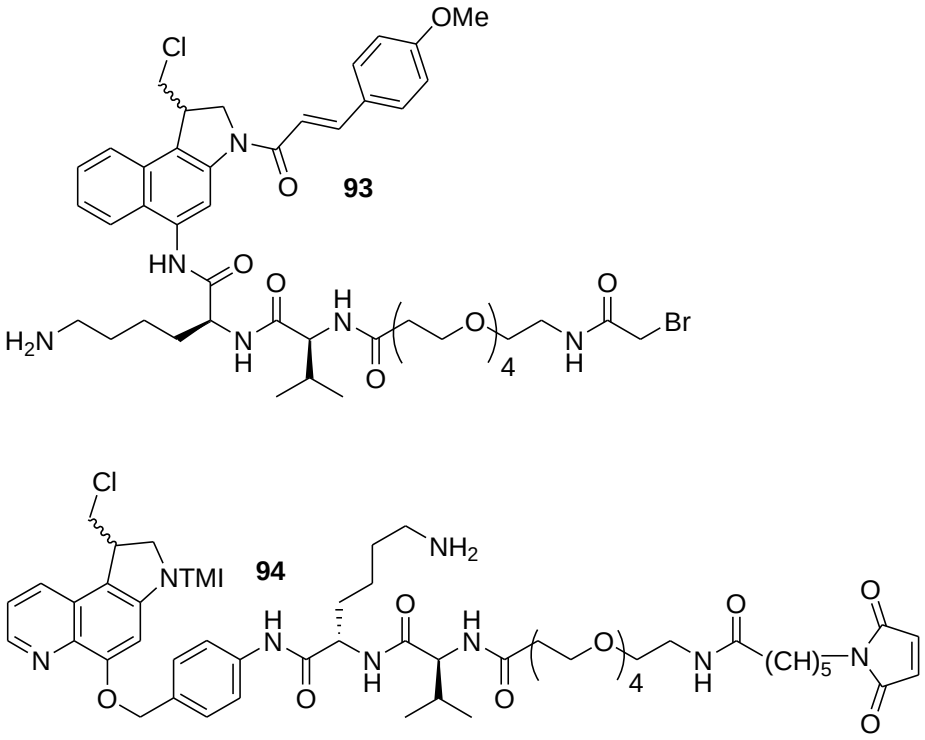


Figure (**51**). *seco*-amino-CBI and *seco*-CpyI conjugates.

The CBI phenol ester prodrug **95** (Fig. **52**) was designed to bind to human serum albumin in the blood stream [154], which would lead to preferential take-up by tumour tissue which has an affinity for human serum albumin (HSA) [155]. The conjugate **95** has reduced cytotoxicity compared to the free phenol (3 x more prodrug could be dosed without causing weight loss, and prodrug-induced weight loss was well-tolerated, no delayed death occurred) and effective in xenograft models. In a colon 38 model, 50% of mice were cured at 0.3 mg/kg with tumour growth inhibition of 99%, significantly better than free phenol (76%) and 5-FU (TGI 95% but no cures). In a SKOV3 model, TGI was 52% compared to 22% with paclitaxel.

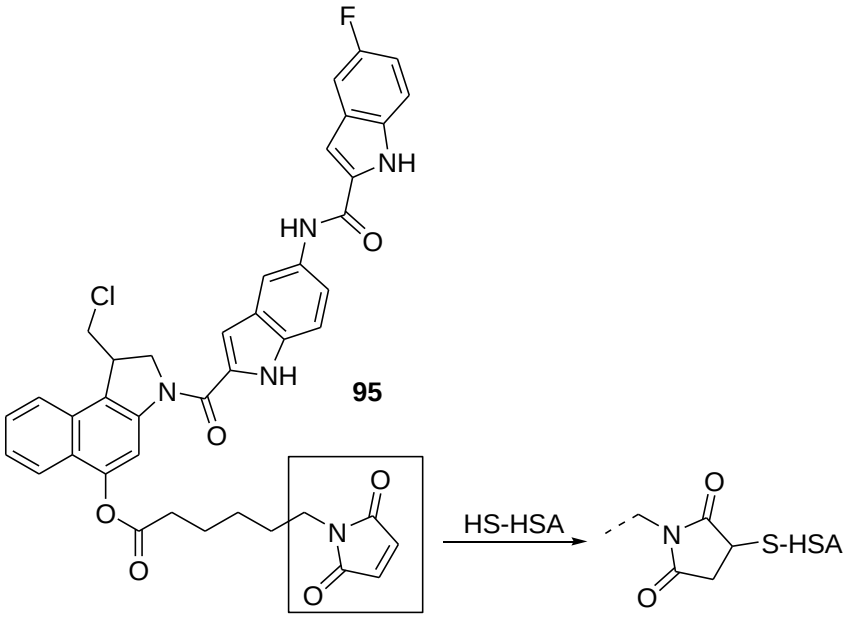


Figure (**52**). CBI-ester prodrug.

CONCLUSION

Due to the intense potency, unique mechanism of action and broad range of antitumour activity of CC-1065 and the duocarmycins, much research has been conducted on these families of natural products since their isolation just over 30 years ago. As highlighted in this review, many research groups around the world still continue to explore new directions in this field. The past decade has added yet another level to our understanding of these unique compounds through many structure-activity relationship studies. Sadly, in spite of these extensive investigations, only four agents have entered clinical evaluation to date – and with disappointing results. However, it is this unmet clinical potential that has driven research groups in this field to perhaps change direction to that of development of prodrug approaches, which aims at selectively releasing potent analogues of CC-1065 and duocarmycins only in malignant tissue. Many investigations have focused on inactivating the

alkylating pharmacophore by masking the chloromethylindoline unit of the *seco*-duocarmycins with electron-withdrawing groups including carbamates on the C6 phenol. Although good differential activity and improved pharmacokinetics in preclinical models have been observed, the clinical trials with carzelesin and KW-2189 have not corroborated this prodrug approach.

The success of prodrug strategies depends on the difference in cytotoxicity between the prodrug and the corresponding cytotoxic metabolite. Accordingly, there is no doubt that the *seco*-duocarmycins lend themselves to being great candidates for use in prodrug strategies due to their structural architecture and inherent cytotoxic potential. This review has highlighted the emerging potential of some of these agents in monotherapy, ADEPT and GDEPT strategies. Fine-tuning these strategies may in the near future lead to drugs that are selectively unmasked in malignant tissue, leading to improved treatment efficacy of patients with solid tumours.

ABBREVIATIONS

ADEPT	Antibody-directed enzyme prodrug therapy
CBA	1,2,9,9a-tetrahydrocyclopropa-[*c*]benz[e]-3-azaindol-4-one
CBI	1,2,9,9a-tetrahydrocyclopropa[*c*]benz[*e*]indol-4-one
CBIn	1,2,9,9a-tetrahydro-1*H*-cyclopropa[*c*]benz[*e*]inden-4-one
CBA	1,2,9,9a-tetrahydrocyclopropa-[*c*]benz[*e*]-3-azaindol-4-one
CBQ	2,3,10,10a-Tetrahydro-1H-cyclopropa[*d*]benzo[*f*]quinol-5-one
CFI	Cyclopropylfurano[2,3-*e*]indoline
CFQ	Cyclopropyltetrahydro-furano[2,3-*f*]quinoline
CI	1,2,7,7a-tetrahydrocyclopropa[*c*]indol-4-one

CNS	Central nervous system
CPI	1,2,8,8a-tetrahydrocyclopropa[*c*]pyrrolo[3,2-*e*]indol-4(5*H*)-one
CPyI	1,2,9,9a-tetrahydrocyclopropa[*c*]pyrido[3,2-*e*]indol-4-one
CPzI	1,2,8,8a-tetrahydrocyclopropa[*c*]pyrazolo[3,2-*e*]indol-4(5*H*)-one
CTI	1,2,8,8a-tetrahydrocyclopropa[*c*]thieno[3,2-*e*]indol-4-one
DHA	Cis-4,7,10,13,16,19-docosahexenoic acid
DNA	Deoxyribonucleic acid
DSA	(+)-Duocarmycin Stable A
ED_{50}	Effective Dose for 50% response
FDI	5-[(5-fluoro-1*H*-indol-2-ylcarbonyl)amino]-1*H*-indol-2-yl]carbonyl
GDEPT	Gene-directed enzyme prodrug therapy
GI_{50}	drug concentration required for 50% Growth Inhibition
HCT	Hydroxycamptothecin
HSA	Human serum albumin
IC_{50}	half maximal Inhibitory Concentration
MeCTI	7-Methyl-1,2,8,8a-tetrahydrocyclopropa[*c*]thieno[3,2-*e*]indol-4-one
MTD	Maximum tolerated dose
NCI	National Cancer Institute
NTR	Nitroreductase
PBD	Pyrrolobenzodiazepine
PEG	Polyethyleneglycol
SAR	Structure-activity relationship
TGI	Tumour growth inhibition
TMI	Trimethoxyindole

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