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Short communication

Does the antitumor cyclopropylpyrroloindole antibiotic CC-1065 cross-link DNA in tumor cells?

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Abstract

We have found that a cyclopropylpyrroloindole antibiotic, compound CC-1065 (benzo[1,2-*b*:4,3-*b′*]dipyrrole-3(2*H*)-carboxamide, 7-[[1,6-dihydro-4-hydroxy-5-methoxy-7-[(4,5,8,8a-tetrahydro-7-methyl-4-oxocyclopropan[c]pyrrolo[3,2-*e*]indol-2(1*H*)-yl)carbonyl]benzo[1,2-*b*:4,3-*b′*]dipyrrol-3(2*H*)-yl]-carbonyl]-1,6-dihydro-4-hydroxy-5-methoxy, (7bR,8aS)), forms interstrand DNA cross-links of an apparently covalent nature in HeLa S_3 cells. This compound induced interstrand cross-links at concentrations ranging from 0.1 to 1 nM/3 hr in whole cells, but these cross-links were absent or marginally low when the drug was added to cell lysates with inactivated cellular enzymes or isolated nuclei, which suggests that metabolic activation of the drug is a necessary step for DNA cross-linking to occur. In contrast, an analog of CC-1065, Bizelesin, which forms DNA–DNA cross-links by direct alkylation, induced interstrand DNA cross-links in both whole cells and in cell lysates. Interestingly, a demethoxy analog of CC-1065, Adozelesin, did not induce DNA cross-links under the same conditions. CC-1065 was found to be extremely potent in terms of concentrations required to cross-link DNA of tumor cells, and this may be related to its remarkable cytotoxic activity.

Keywords: CC-1065; Interstrand DNA cross-links; Cytotoxicity; Metabolic activation

1. Introduction

CC-1065 (benzo[1,2-*b*:4,3-*b′*]dipyrrole-3(2*H*)-carboxamide, 7-[[1,6-dihydro-4-hydroxy-5-methoxy-7-[(4,5,8,8a-tetrahydro-7-methyl-4-oxocyclopropan[c]pyrrolo[3,2-*e*]indol-2(1*H*)-yl)carbonyl]benzo[1,2-*b*:4,3-*b′*]dipyrrol-3(2*H*)-yl]-carbonyl]-1,6-dihydro-4-hydroxy-5-methoxy, (7bR,8aS)), a cyclopropylpyrroloindole antibiotic isolated from *Streptomyces zelensis* [1], is one of the most cytotoxic antitumor agents known. This antibiotic has a unique chemical structure with a cyclopropyl group capable of alkylating DNA and a half-moon shaped backbone of the molecule, which enables a fit in the minor groove of DNA (for review, see Refs. [2] and [3]); these two features of the drug are believed to be responsible for its cytotoxic and antitumor activity. The drug causes, however, an undesirable delayed liver toxicity in mice, which prevented it from being used in clinical applications, and many analogs have been synthesized to overcome this effect [4–6]. Two analogs of CC-1065, Bizelesin and Adozelesin, have entered clinical studies.

Compounds that monoalkylate DNA usually do not exert potent antitumor properties. However, CC-1065 has been shown to bind selectively to AT-rich sequences of DNA [7,8], and this feature has been proposed to be responsible for its very high cytotoxic activity. The non-distorting nature of DNA lesions induced by CC-1065 suggests that its DNA adducts may represent intractable lesions to DNA repair enzymes. In line with this, it has been shown that CC-1065-induced DNA lesions are not readily reversible in BSC-1 green monkey cells [9]. On the other hand, DNA adducts induced by minor groove binding compounds with alkylating potency, including CC-1065, are recognized by tumor cells [10], and in this respect the extremely high cytotoxic and antitumor potency of CC-1065 is still far from clear.

We hypothesized that CC-1065 may undergo metabolic activation by cellular enzymes and, apart from monoalkylation by the cyclopropyl group, may be able to form an additional covalent bond with DNA and thereby induce DNA–DNA cross-links. This hypothesis was evaluated by a newly developed fluorimetric method for DNA interstrand

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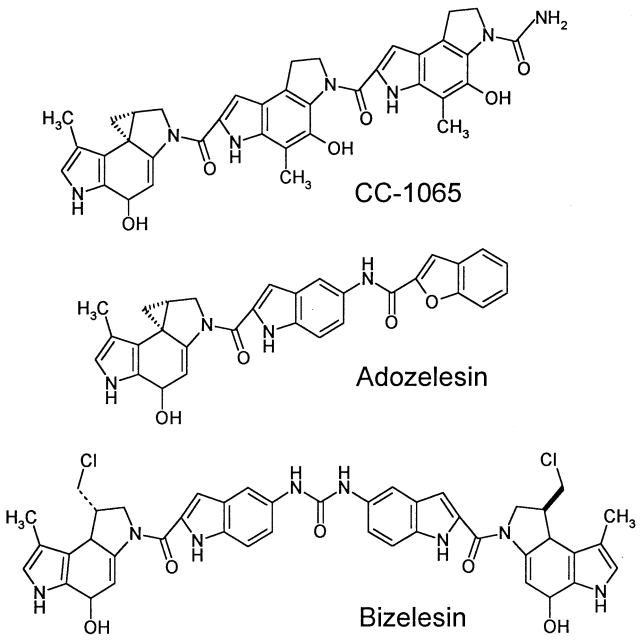

Fig. 1. Chemical structures of CC-1065 and its analogs.

cross-link measurement. We compared the DNA cross-linking capability of CC-1065 with that of its analogs: Bizelesin, which forms DNA cross-links by direct chemical bonding, and Adozelesin, a demethoxy derivative of CC-1065.

2. Materials and methods

2.1. Drugs

CC-1065 (U-56,314) and its analogs, Bizelesin (U-77,779) and Adozelesin (U-73,975) (see Fig. 1 for chemical structures), were provided by Dr. Patrick McGovren (Upjohn). The drugs were dissolved in dimethylacetamide at 1 mg/mL and stored at −20° until used.

2.2. Chemicals

Ethidium bromide homodimer was synthesized in our Department according to a published method [11], and its properties and purity were ascertained by comparison to the compound provided by Prof. Bernard Roques (University of Paris). PicoGreen was from Molecular Probes, and all other reagents were from the Sigma Chemical Co.

2.3. Cell culture and medium

HeLa S_3 cells, medium, and serum were purchased from Gibco Europe Ltd.; antibiotics were obtained from SERVA. The cells were grown in a monolayer culture in minimal essential medium with 5% fetal bovine serum and antibiotics (streptomycin, 100 μg/mL; penicillin, 100 U/mL) at 37° in a humidified 5% CO_2-95% air atmosphere.

2.4. Cytotoxicity assay

The cytotoxic activity of studied drugs toward HeLa S_3 cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells (2×10^4 per well) were allowed to attach in 24-well plates for 24 hr before a 3-hr exposure to drugs. Following two washes with warm growth medium, cells were incubated for an additional 96 hr. The IC_{90} is defined as the inhibitory drug concentration causing 90% reduction of A_{540} absorbance versus that of control.

2.5. DNA interstrand cross-linking assay in intact cells and isolated nuclei

The technique relies on fluorescence measurements of a complex between ethidium dimer and double-stranded DNA after heat denaturation and renaturation of DNA from cells treated with the drug. Heat denaturation of cross-linked DNA is prevented, which results in higher fluorescence of the ethidium dimer–DNA complex compared with non-cross-linked DNA.

Upon completion of the incubation time, 2.5 x 10^6 cells, either control or treated with drugs, were washed twice with ice-cold PBS, resuspended in 100 μL PBS, and mixed with 300 μL of lysing solution (50 mM Tris–HCl, pH 8, 10 mM EDTA, 1% lauroyl sarcosine, 0.5 mg/mL of proteinase K, final concentrations) and incubated at 37° for 2 hr with occasional mixing. Following lysis, 3.6 mL of denaturing solution was added (6 M sodium perchlorate, 1 mM EDTA, 0.2% lauroyl sarcosine, 20% methanol, pH 7) and mixed thoroughly. The lysates were left at room temperature for 1 hr and divided into ‘native’ non-denatured and ‘denatured’ samples (1 mL each), and DNA was denatured by heating ‘denatured’ samples in a water bath at 50° for 30 min and renatured by rapid dilution with 5 mL of 0.1 M Tris–HCl buffer, pH 8, and cooling in an ice–methanol mixture (−18°) for 1 min. The ‘native’ samples were diluted with 5 mL of Tris–HCl buffer without heating, 0.5 mL of each cell lysate was added to 4.5 mL of phosphate buffer (20 mM potassium phosphate, 0.5 mM EDTA, pH 11.9), mixed, and diluted 1:1 with ethidium dimer solution (0.4 μg/mL in phosphate buffer). The samples were incubated for 15 min at room temperature in the dark before fluorescence was measured by a Perkin-Elmer LS-5B luminescence spectrometer with excitation and emission wavelengths of 540 and 595 nm, respectively. In some experiments, a different fluorochrome (PicoGreen) was used, which does not require estimation of background fluorescence due to its higher specificity for double-stranded DNA and negligible background fluorescence in whole cell lysates [12]. A solution of PicoGreen (1:200 dilution of commercial stock in 10 mM Tris–HCl, 1 mM EDTA, pH 7.5) was mixed 1:1 with samples, and fluorescence was determined with excitation and emission wavelengths of 480 and 520 nm, respectively.

For the cell-free system, untreated cells were lysed as

described above, and drugs were added at the specified concentrations. After thorough mixing and incubation of cell lysates in the dark for 3 hr at 37°, DNA cross-linking was determined as described above. DNA cross-linking also was determined in isolated nuclei prepared as described [13]. Briefly, cells were washed twice in cold PBS and once with nuclei isolation buffer (2 mM potassium phosphate buffer, pH 7.0, 5 mM magnesium chloride, 150 mM sodium chloride, 1 mM EDTA, 0.2 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 10% glycerol) at 4°. The cell pellet was resuspended in the same buffer and lysed in the presence of 0.3% Triton X-100 for 10 min on ice. Nuclei were harvested by centrifugation (1000 *g*, 10 min), washed twice in nuclei isolation buffer, and resuspended at 1×10^6 nuclei/mL in the same buffer and exposed to drugs for 3 hr at 37°. Following drug treatment, nuclei were processed as described above for whole cells.

To determine blank fluorescence for all the samples, both 'native' and 'denatured' samples were heated at 95° for 15 min, rapidly quenched in an ice–methanol mixture for 1 min, and warmed to room temperature, and residual fluorescence was determined as described above. These fluorescence values were subtracted from the total fluorescence of all lysates to obtain values deriving exclusively from the complex of double-stranded DNA and ethidium dimer. Typical f_d/f_n values for samples from non-treated cells were 0.1 to 0.15 (N = 27).

Ratios of fluorescence of 'denatured' and 'native' samples from treated and control cells, $(f_d/f_n)_t$ and $(f_d/f_n)_c$, respectively, were converted into the number of cross-links per 10^6 bp according to a method described earlier [14], assuming the size of the human genome to be 2.8×10^9 bp, the average size of DNA in cell lysates to be 2×10^5 bp, and a random distribution of DNA cross-links.

2.6. Thermal and alkaline stability of DNA cross-links

Cell lysates from drug-treated cells were either heated at 95° for 15 min or alkalinized by the addition of sodium hydroxide to 30 mM and incubated for 1 hr at room temperature followed by neutralization with hydrochloric acid. Samples then were processed for DNA cross-linking determination as described above.

3. Results and discussion

In this work, we elucidated whether the antibiotic CC-1065 is able to form interstrand DNA cross-links in HeLa S_3 cells. For DNA cross-link measurements, we used a new fluorimetric method that allows determination of DNA interstrand cross-links unstable to heat and alkali. The method used is a variant of one described previously [15] in which cellular DNA is denatured in mild conditions (6 M sodium perchlorate, 50°, 30 min) avoiding elevated temperatures and high pH, and the fraction of renatured DNA is determined by a nuclease S1 assay. Nuclease S1 digestion is inhibited in the presence of compounds with high affinity to DNA, such as CC-1065 and its analogs, and this assay potentially could be a source of artifacts (our unpublished observations). In the new method, the fractions of renatured DNA were determined by fluorimetric measurements using ethidium homodimer or PicoGreen, two dyes with high affinity for double-stranded DNA [12,16]. We used these fluorochromes since in our method a high concentration of sodium perchlorate for DNA denaturation is used, which lowers the temperature of DNA denaturation. The latter compound quenches fluorescence, and cell lysates should be diluted at least 100-fold to avoid this effect.

Using our method, we found that CC-1065 caused a concentration-dependent increase in the number of cross-links in DNA from cells treated with the drug (Fig. 2). We calculated by a linear fit the relationship between the number of DNA cross-links and the concentration of the drug, concentration C_0, corresponding to the concentration at which the first DNA cross-link could be observed by our method. We used this concentration to characterize DNA cross-linking potency of the drugs studied. We observed interstrand DNA cross-linking for CC-1065 only in the cellular system, with no increase of the number of DNA cross-links when the drug was added directly to cellular lysates in which enzymes had been inactivated by detergent and a high concentration of sodium perchlorate (Fig. 2). It should be stressed that drug:DNA ratios in cell lysates were comparable to or even higher than those in whole cells. Additionally, we assessed DNA cross-linking induced by CC-1065 in isolated nuclei from untreated cells (Fig. 3). CC-1065 induced a very low level of DNA cross-linking in isolated nuclei, only about 10–20% of that observed in whole cells. The low but measurable DNA cross-linking in isolated nuclei, especially at the highest concentration tested, could be attributed to the activity of nuclear enzymes. It has been shown that at least some drug-metabolizing enzymes, such as peroxidases or cytochrome P450 enzymes, may be present in the nuclear membrane, nucleoplasm, or even bound to chromatin (e.g. Refs. [17] and [18]). The absence or marginally low levels of DNA cross-linking in cell lysates and isolated nuclei suggests that metabolic activation is a necessary step leading to DNA cross-linking by CC-1065. These results also suggest that the observed increased renaturation of DNA from cells treated with CC-1065 did not result from strong physicochemical binding of the drug to DNA.

We compared DNA cross-linking by CC-1065 with a similar property of its analog, Bizelesin, which possesses two chloromethyl groups that can be converted into cyclopropyl moieties and is thus able to bind bifunctionally to DNA without metabolic activation [5,6,19]. This compound formed DNA cross-links in HeLa S_3 cells in a concentration-dependent manner (Fig. 2). In contrast to CC-1065, Bizelesin was also able to cross-link DNA of HeLa S_3 cells when added directly to lysates from untreated cells (Fig. 2).

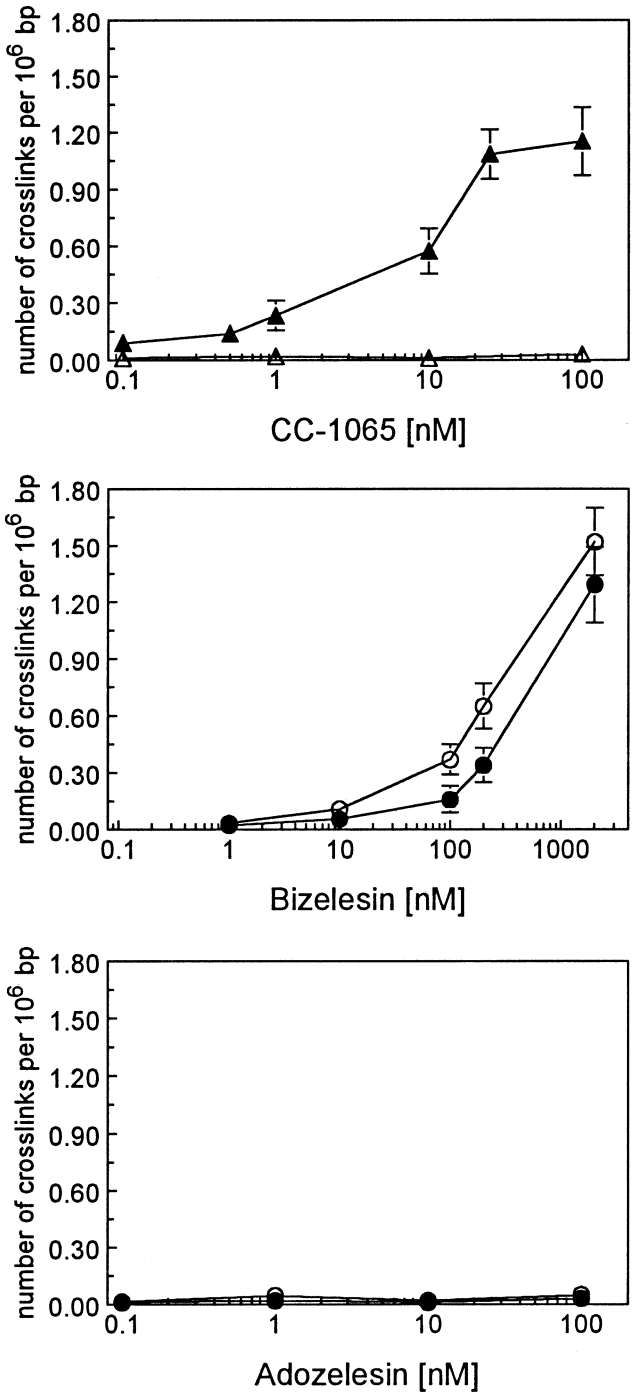

Fig. 2. DNA cross-linking induced by CC-1065 and its analogs in HeLa S_3 cells (closed symbols) and cellular lysates (open symbols). Whole cells or cellular lysates were treated with the drugs for 3 hr at 37°, and DNA cross-linking was determined as described in 'Materials and methods'. Values are means ± SD (N = 3–5).

Interestingly, the DNA cross-linking potential of Bizelesin was slightly higher in cell-free conditions, in terms of the concentration required to detect the first DNA cross-link: the C_0 concentration equaled 11 nM in whole cells and 7 nM in the cell-free system. Bizelesin probably binds to a higher extent to DNA in cellular lysates due to a better

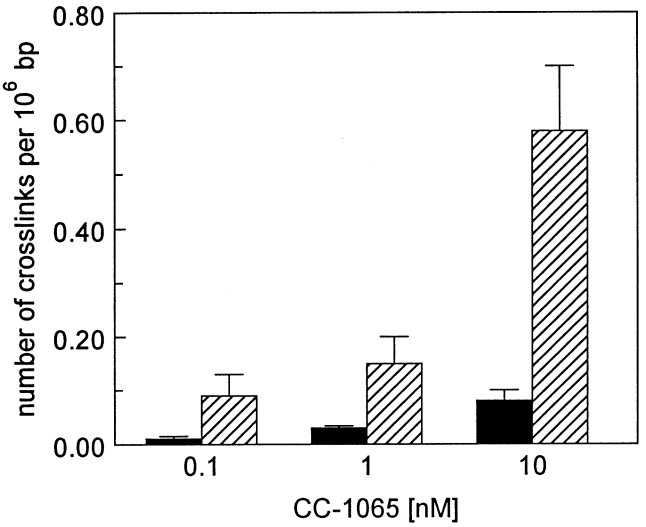

Fig. 3. DNA cross-linking induced by CC-1065 in HeLa S_3 cells (hatched bars) and isolated nuclei (closed bars). Whole cells and nuclei were incubated with the drug for 3 hr at 37°, and DNA cross-linking was determined as described in 'Materials and methods'. Values are means ± SD (N = 3).

accessibility of cellular DNA, partially relaxed in the lysing buffer, or, alternatively, there might be less detoxification of Bizelesin in cell lysates with inactivated enzymes. We also tested a demethoxy analog of CC-1065, Adozelesin, for its DNA cross-linking potency and observed no effect at any concentration used (Fig. 2).

DNA cross-links formed by CC-1065 were unstable at elevated temperatures and at alkaline pH, since we did not observe any measurable DNA cross-linking by this compound when determined by another fluorimetric method [20] with DNA denaturation by heating at pH 12 and at 95° for 5 min. We also exposed cell lysates from drug-treated cells to alkali (pH 12, 1 hr, room temperature) before the DNA denaturation/renaturation cycle, and observed marginal renaturation of DNA. Similarly, we determined the thermal instability of DNA cross-links formed by CC-1065 by heating cell lysates (95°, 15 min, pH 7) and observed no DNA cross-linking (data not shown). It has been shown previously that the chemical bond formed between N3 of adenine and the cyclopropyl group of CC-1065 is thermally unstable [6]. In addition, DNA cross-links formed by Bizelesin are also unstable when exposed to alkaline conditions for prolonged periods of time [21] and cannot be found by alkaline elution at biologically relevant concentrations [22]. DNA cross-links induced by Bizelesin, however, resist shorter alkaline treatment and can be detected at nanomolar concentrations by alkaline sucrose gradient centrifugation [19].

CC-1065 was about 13-fold more cytotoxic toward HeLa S_3 cells than its analog, Bizelesin. This difference in cytotoxicity was accompanied by a higher DNA cross-linking capability of CC-1065 in HeLa S_3 cells than that of Bizelesin (Table 1). This result and the fact that for CC-1065 DNA cross-linking could be observed at cytotoxic concen-

Table 1
Cytotoxic activity and DNA cross-linking of CC-1065 and its analogs in HeLa S_3 cells determined after a 3-hr treatment with drugs

Compound	IC_{90}[a] (nM)	C_o[b] (nM)
CC-1065	0.058 ± 0.07	0.078 ± 0.04
Adozelesin	0.426 ± 0.03	-
Bizelesin	0.72 ± 0.6	11.0 ± 2
Mitomycin C	4430 ±t50	10,800 ± 620

Values are means ± SD from at least three independent experiments.
[a] Concentration inhibiting cell growth by 90% compared with non-treated cells.
[b] Concentration at which the first DNA cross-link could be detected.

trations (compare IC_{90} and C_0 concentrations in Table 1) suggest that there is a relation between biological activity and DNA cross-linking by this drug; however, further studies are required to prove this notion. It is worthwhile noting that DNA cross-linking by CC-1065 was observed at extremely low concentrations, considerably lower than for mitomycin C, a classical DNA alkylator (Table 1).

Since CC-1065 induces formation of DNA monoadducts, a question remains as to what is the proportion of DNA cross-links in the total DNA lesions induced by this drug. Although we did not determine the total DNA damage induced by CC-1065 in HeLa S_3 cells, it could be estimated roughly, based on published data by Zsido et al. [9]. In BSC-1 green monkey cells, CC-1065 induces 3–300 adducts/10^6 bp after a 2-hr treatment with 3–300 nM drug. Assuming that the total DNA damage is comparable in HeLa S_3 cells, DNA cross-links constitute about 10–15% of all DNA lesions induced by CC-1065 in these cells. It follows that a majority of the DNA damage induced by CC-1065 in HeLa S_3 cells are monoadducts. Further studies clearly are required to fully explain the proportion of different types of DNA damage induced by CC-1065, including a new effect of this drug, which is DNA cross-linking.

In the chemical structure of CC-1065, there is only one reactive group that can bind DNA, the reactive cyclopropyl ring. The second bond required for DNA cross-linking by CC-1065 should be formed by enzymatic activation of the parent drug. On the basis of the available literature, we can propose a possible pathway of metabolic activation that leads to formation of DNA cross-links (Fig. 4). According to this mechanism, enzymatic demethylation of a methoxy group in *o*-methoxy-phenol moieties yields *o*-quinone. An *o*-methoxy-phenol moiety is present in a variety of biologically active compounds, including antitumor agents (e.g. the epipodophyllotoxin derivative VP-16) and is metabolized to *o*-quinone through *O*-demethylation [23]. This group has been shown to be involved in drug binding to nucleophilic centers of cellular macromolecules (for review see Ref. [24]). It should be noted that in the chemical structure of CC-1065 there are two *o*-methoxy-phenol groups that potentially can be transformed into their respective *o*-quinones. A lack of DNA cross-linking by Adozelesin, a demethoxy analog of CC-1065, further strengthens the potential role of the *o*-methoxy-phenol group in DNA cross-linking by CC-1065. Alternatively, a second mechanism can be proposed in which the activated indole moiety of CC-1065 reacts with 5,6-dihydroxy or 5,6-quinone intermediates, as was shown for precursors of melanins [25]. The proposed pathways leading to formation of DNA cross-links

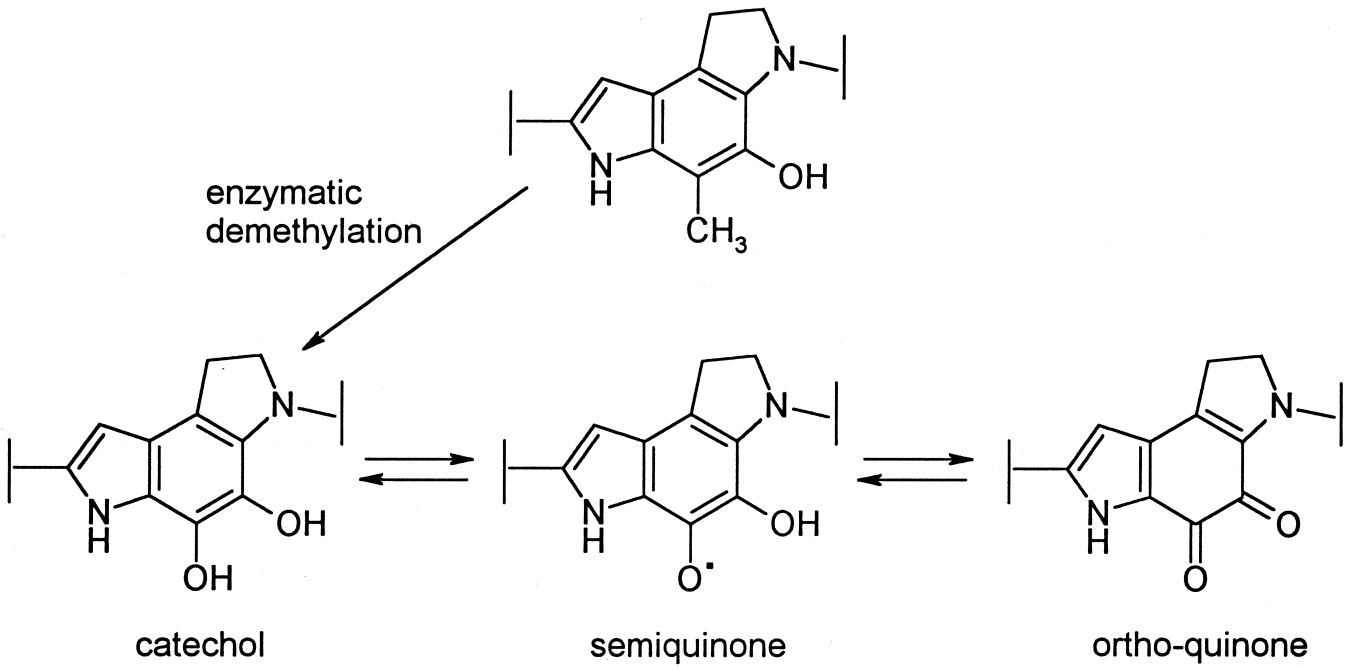

Fig. 4. A putative pathway of metabolic activation of CC-1065.

by CC-1065 are likely, but still speculative, and remain to be proved experimentally.

In conclusion, we show in the present study that a very cytotoxic cyclopropylpyrroloindole drug, CC-1065, forms interstrand cross-links in HeLa S_3 cells. Due to the instability of the chemical bond(s) involved in DNA cross-linking by CC-1065, this effect could not have been detected so far by conventional methods used for DNA cross-link measurements in which high temperature and/or alkaline pH is used. Metabolic activation of CC-1065 was necessary for DNA cross-linking by this compound. The data presented indicate that CC-1065, along with its analog Bizelesin, pyrrolodiazepines [2], and derivatives of neotropsin [26], is a member of a new class of minor groove binders and DNA cross-linking agents. In contrast to other compounds from this group, such as Bizelesin, CC-1065 should be metabolized to form cross-links with cellular DNA.

Acknowledgments

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