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CD-Spectroscopy As a Powerful Tool for Investigating the Mode of Action of Unmodified Drugs in Live Cells

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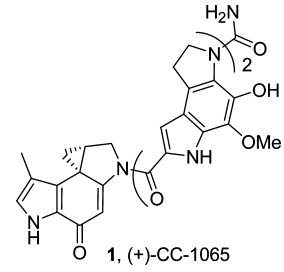
Abstract: Circular dichroism (CD) spectroscopy is a well-known method for the analysis of chiral chemical compounds and is often used for studying the structure and interaction of proteins, DNA and bioactive compounds in solution. Here we demonstrate that CD spectroscopy is also a powerful tool for investigating the cellular uptake and mode of action of drugs in live cells. By means of CD spectroscopy, we identified DNA as the cellular target of several novel anticancer agents based on the highly cytotoxic natural antibiotic CC-1065. Furthermore, time-dependent changes in the CD spectra of drug-treated cells enabled us to rationalize differences in drug cytotoxicity. The anticancer agents rapidly penetrate the cell membrane and bind to cellular DNA as their intracellular target. Thereby, the formation of a reversible noncovalent complex with the DNA is followed by a covalent binding of the drugs to the DNA and the more toxic compounds show a higher stability and a lower alkylation rate. Since no drug manipulation is necessary for this kind of investigation and achiral compounds bound to chiral biomolecules may also show induced CD signals, CD spectroscopy of live cells is not limited to the study of analogues of CC-1065. Thus, it constitutes a general approach for studying the mode of action of bioactive compounds on the cellular and molecular level.

Introduction

Circular dichroism (CD) is based on the differential absorption of polarized light by chiral molecules or complexes and is usually measured as ellipticity. The wavelength-dependent CD is a characteristic feature for a given compound and is used to determine its absolute configuration.[1] Moreover, CD spectroscopy is a convenient technique to investigate conformational changes, secondary structures of biomolecules, and intermolecular interactions.[1,2] The formation of covalent or noncovalent complexes of chiral or nonchiral compounds with macromolecules like peptides, proteins, DNA, or RNA can lead to a strong induced CD (ICD) signal characteristic for the interaction.[1−3] The latter is caused by a coupling of electric transition moments of the ligand and the biomolecule in the asymmetric binding site. For example, the CD of a mixture of double-stranded DNA and the natural antibiotic CC-1065 (**1**, Figure 1)[4] or its analogues reveals a strong induced CD in solution, which can be rationalized by a binding of the compounds to DNA.[5]



1, (+)-CC-1065

Figure 1. Structure of the natural antibiotic (+)-CC-1065 (**1**).

CC-1065 (**1**) has a very high cytotoxicity, with an IC_{50} of about 20 pM (leukemia cell line L1210) and several analogues of **1** have entered clinical trials as anticancer agents.[5,6] The proposed mode of action of these compounds is a sequence-selective binding of the drugs to the minor groove of cellular DNA with the cyclopropyl moiety acting as an alkylating unit.[5−7] As a consequence, important cellular functions are affected and cell death is induced. We have developed a series of glycosidic, comparably little toxic prodrugs such as **2a**−**5a**

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of the corresponding highly cytotoxic *seco*-analogues **2b**−**5b** of CC-1065 (**1**) for use in antibody-directed enzyme prodrug therapy[8] and prodrug monotherapy[9] of cancer (Figure 2).[10]

The detoxifying sugar units of these prodrugs are cleaved, employing the corresponding glycohydrolase to give the *seco*-drugs **2b**−**5b**, which rapidly undergo an in situ-cyclization under loss of HCl to provide the corresponding highly reactive drugs **2c**−**5c** containing a cyclopropyl moiety like in **1**. Since these drugs show a high reactivity toward synthetic double-stranded DNA, we assume that their binding to cellular DNA (Scheme 1) is responsible for their high cytotoxicity.[11]

As described here, the *seco*-drugs **2b**−**5b** show a pronounced induced CD when they are incubated with DNA in solution. On the basis of this observation, we performed CD spectroscopy on drug-treated live cells using cell suspensions and a common CD spectrometer. As such, we were able to investigate the cellular uptake and mode of action of the unmodified drugs. Monitoring changes in CD spectra after exposure of live cells to drugs has, so far, not been described.[12] Importantly, CD spectroscopy of cells allows the study of a drug's mode of action without prior drug manipulation; this is a strong advantage compared to established methods such as labeling with radioactive isotopes[13] or fluorescent dyes.[14] Furthermore, since CD is sensitive to structural changes of molecules, analyses of the CD spectra can contribute to a reasonable rationalization of the mode of action and of differences in biological activity. The understanding of the mode of action of drugs is very important for their later administration and may enhance therapeutic efficiency. Moreover, it can have a high impact on drug development.[15]

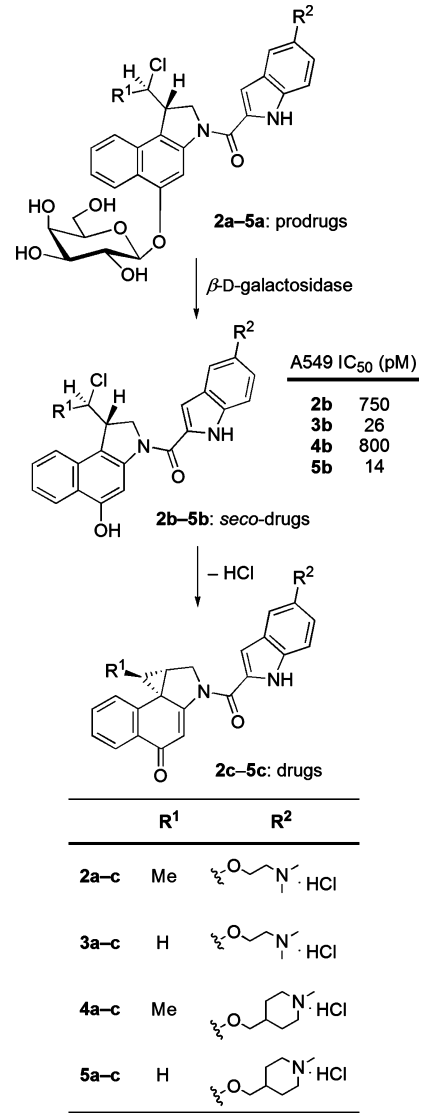

Figure 2. Prodrugs **2a**−**5a**, their enzymatic transformation into the *seco*-drugs **2b**−**5b** followed by a fast cyclization to give the corresponding drugs **2c**−**5c**. Cytotoxic activity of the novel *seco*-drugs **2b**−**5b** against human bronchial carcinoma cells of line A549.

Results and Discussion

CD Spectra of *seco*-Drugs 2b−5b in Buffer with and without DNA. In the absence of DNA, the *seco*-drugs show a weak negative CD at $\lambda = 250$ nm and weak positive CD signals at $\lambda = 275$, 320, and 390 nm (Figure 3).

The CD signals change in time due to a fast cyclization of the *seco*-drugs **2b**−**5b** to afford the corresponding drugs **2c**−**5c** containing a spirocyclopropyl moiety as in CC-1065 (**1**). Thereby, the CD signal at $\lambda = 390$ nm is characteristic for the *seco*-forms. Employing **4b** and **5b**, containing a morpholinoethoxy substituent, this CD signal is visible for up to 60 min after starting the incubation. In contrast, the *seco*-drugs **2b** and **3b**, containing the *N*,*N*-dimethylaminoethoxy moiety, do not show this signal because they cyclize nearly instantaneously to form the corresponding drugs in buffered solution.

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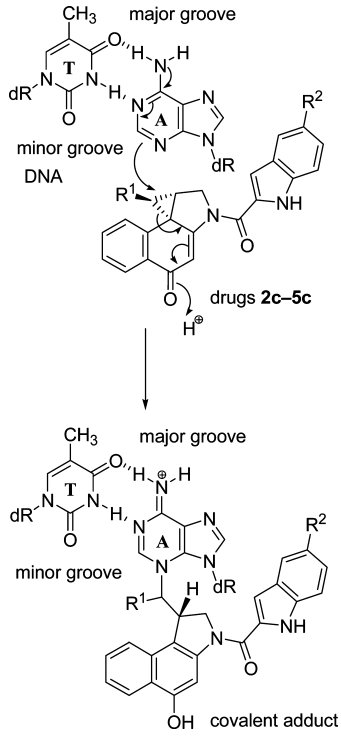
Scheme 1. Reaction between the Highly Reactive Drugs **2c**−**5c** and Double-Stranded DNA



In eukaryotic cells, the nuclear double-stranded DNA is organized in the form of chromatin by an interaction with RNA and proteins in order to package the large DNA in a smaller volume and to facilitate and control transcription as well as replication and cell division. A very important structural element of chromatin is the nucleosome core particles that consist of 1.67 turns of superhelical DNA (146 base pairs) wrapped around a histone octamer.[16] All nucleosome core particles are connected to the adjacent ones by free DNA of 10 to 80 base pairs, and the resulting fiber of nucleosomes and linker DNA is coiled again to form helical filaments and other higher order structures.[17] Trzupek et al. have investigated the binding of CC-1065 analogues to free DNA (α-satellite DNA) as compared to the same DNA wrapped around histones to form nucleosome core particles.[7a] Thereby, they observed a similar alkylating efficiency and sequence selectivity for both kinds of DNA,

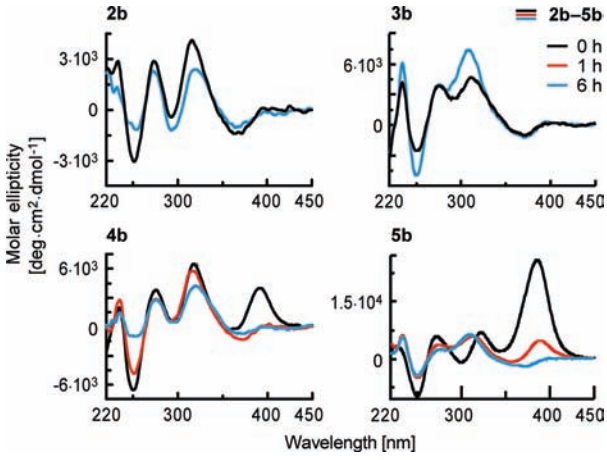


Figure 3. Molar ellipticity of a solution of the *seco*-drugs **2b**−**5b** in phosphate buffered saline after the indicated times of incubation at 25 °C.

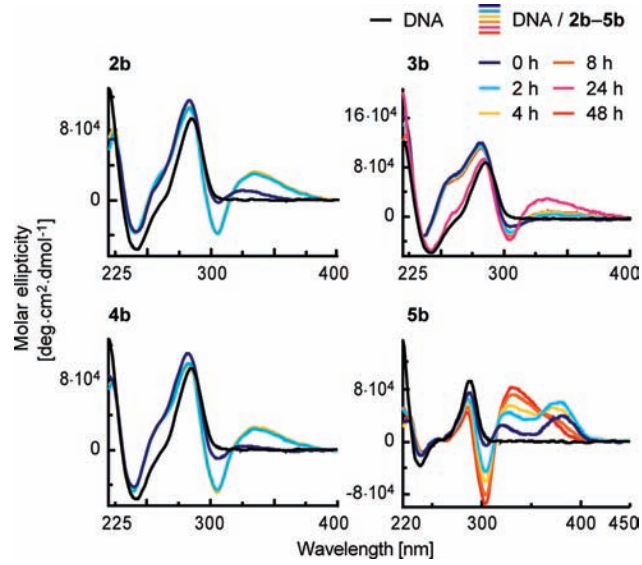


Figure 4. Molar ellipticity of mixtures of the synthetic DNA oligonucleotide 5′-d(CGGTCAATTAGT-CGC)-3′ · 3′-d(GCCAGTTAATCAGCG)-5′ and *seco*-drugs **2b**−**5b** in phosphate buffered saline.

irrespective of the distortions in the DNA structure upon packaging in nucleosome core structures. On the basis of these results, they concluded that at least for these kinds of drugs, cell- and protein-free DNA can be considered as a good model for DNA in eukaryotic cells. Due to their different organization and three-dimensional structure, chromatin and free DNA show slightly different CD spectra. However, the binding of CC-1065 and its analogues to chromatin and free DNA induces very similar CD-bands with nearly identical minima and maxima,[18] making free DNA also a good model for nuclear DNA regarding studies of induced CD signals caused by the interaction of CC-1065 analogue drugs with DNA. Furthermore, since the induced CD signals of free DNA and synthetic DNA oligonucleotides are very similar regarding the minima and maxima of the induced CD bands[19] and, in addition, the synthetic oligomers have a defined structure and can be produced in high purity and reproducible quality, we chose synthetic DNA oligonucleotides as the model structures for investigations of the interaction of our novel drugs with DNA.

The CD spectra of mixtures of the *seco*-drugs **2b**−**5b** with synthetic double-stranded DNA oligonucleotides such as 5′-d(CGGTCAATTAGTCGC)-3′ · 3′-d(GCCAGTTAATCAGCG)-5′ containing an AT-rich region show a characteristic induced CD with a minimum at $\lambda = 305$ nm and a maximum at $\lambda = 335$ nm (Figure 4, colored lines) that cannot be observed for the DNA oligonucleotides alone (Figure 4, black lines).

The ICD is much stronger than the CD observed for the *seco*-drugs in the absence of DNA and its increase with incubation time reflects the binding of the drugs **2c**−**5c** to the DNA oligonucleotides. Employing the *seco*-drugs **2b** and **4b**, the maximum intensity of the induced CD is reached after 2 h

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(Figure 4), indicating a fast binding of the corresponding drugs **2c** and **4c**, respectively, to the DNA oligonucleotides. In contrast, using the *seco*-drugs **3b** and **5b**, the maximum intensity is reached after 24 and 48 h, respectively, indicating a much slower binding of the corresponding drugs **3c** and **5c** to the DNA oligonucleotides.

The lower reaction rate of **3b** and **5b** with DNA oligonucleotides as compared to **2b** and **4b** can be explained by the higher stability of the unsubstituted cyclopropyl moiety in **3c** and **5c**, which is formed in situ from **3b** and **5b**, toward a nucleophilic attack. The lower reaction rate of **5b** in comparison to **3b** seems to be caused by a partial stabilization of the *seco*-form **5b** by an interaction with the double-stranded DNA oligonucleotides. As a consequence, a positive CD at $\lambda = 390$ nm can be observed for as long as 8 h after starting the incubation of **5b** with the DNA oligonucleotides. Furthermore, the interaction of **5b** with the DNA oligonucleotides induces an increase of the intensity of the signal at $\lambda = 390$ nm in the first 2 h after starting the incubation. The following decrease of its intensity is accompanied by an increase of the induced CD characteristic for the binding of the drug to the DNA oligonucleotides, showing a minimum at $\lambda = 305$ nm and a maximum at $\lambda = 335$ nm. Thus, a covalent binding of the drug to the DNA oligonucleotides takes place after a preliminary interaction of the *seco*-drug with the oligonucleotide, followed by a cyclization of the *seco*-drug to afford the reactive drug. Whereas *seco*-drug **3b** shows a similar but less intense stabilization of the *seco*-form when it is incubated with double-stranded DNA oligonucleotides of base sequences that differ from the sequence shown here, the *seco*-drugs **2b** and **4b** do not reveal an analogue stabilization by neither of the investigated DNA oligonucleotides.

CD Spectroscopy of *seco*-Drug-Treated Cells. Since all *seco*-drugs caused a strong induced CD after binding of the corresponding in situ formed drugs to double-stranded DNA oligonucleotides in solution, we examined whether this characteristic behavior could be used for the investigation of the mode of action of the *seco*-drugs in live cells. Especially, we were interested in whether a binding of the drugs to DNA occurred only in buffered solution or also in live cells and thus could be responsible for the high biological activity of these anticancer agents.

For the CD investigations on cells, tumor cells of the human promyelocytic leukemia cell line HL-60 were incubated with the *seco*-drugs **2b**−**5b** for several hours. At selected points in time, the cells were washed, resuspended in PBS, and CD spectra of suspensions of the treated cells in PBS were recorded (Figure 5). Besides the unspecific CD signals below $\lambda = 300$ nm caused by cellular proteins and cellular DNA, the spectra show an induced CD with a minimum at $\lambda = 305$ nm and a maximum at $\lambda = 335$ nm (Figure 5, colored lines). Such an induced CD, as discussed before, is characteristic for the interaction between DNA oligonucleotides with the drugs **2c**−**5c** that are formed in situ from the *seco*-drugs **2b**−**5b**, and based on previous results,[17,18] it can be assumed that the ICD reflects the binding of the drugs to chromatin as well. Accordingly, an induced CD was not observed for cells that were not exposed to the *seco*-drugs (Figure 5, black lines).

The results strongly indicate that a binding to the cellular DNA is indeed the mode of action of the drugs in live cells. Besides the induced CD characteristic for the binding of the drugs to DNA, cells incubated with the *seco*-drugs **3b** and **5b** show an additional maximum at $\lambda = 390$ nm that is characteristic for the *seco*-forms. This indicates a stabilization of the *seco*-drugs **3b** and **5b** inside the cells.

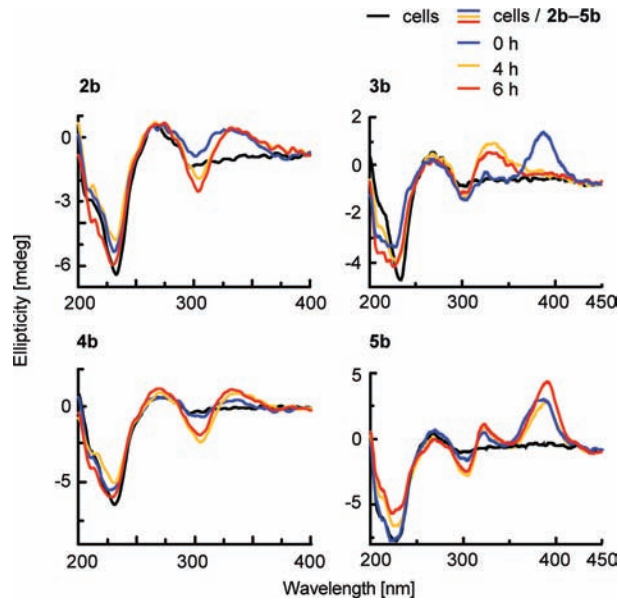

Figure 5. Ellipticity of suspended cells after exposure to the *seco*-drugs **2b**−**5b** for the time indicated.

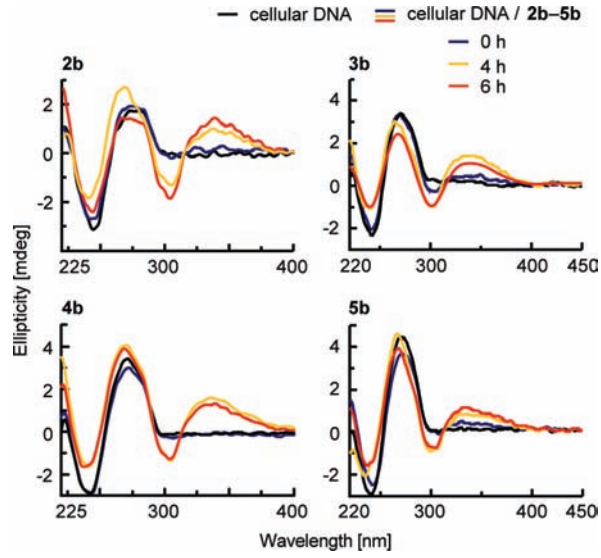

Figure 6. Ellipticity of cellular DNA after exposure of cells to the *seco*-drugs **2b**−**5b** for the time indicated.

CD Spectroscopy of DNA Isolated out of *seco*-Drug-Treated Cells. To confirm that the induced CD observed after incubation of cells with the *seco*-drugs is caused by a binding of the drugs to the cellular DNA, we isolated and purified the DNA of the cells after incubation with **2b**−**5b**. The CD-spectra of the isolated DNA show the same characteristic induced CD signals as observed for the cells incubated with **2b**−**5b** (Figure 6, colored lines), whereas DNA isolated from untreated cells shows no induced CD (Figure 6, black lines). Furthermore, since noncovalently bound species were washed out of the DNA during the DNA purification steps, no CD at $\lambda = 390$ nm characteristic for the *seco*-drugs was observed.

In addition, from differences in the CD-spectra of *seco*-drug-treated cells and the DNA isolated out of these cells, it can be deduced that the administered *seco*-drugs penetrate the mem-

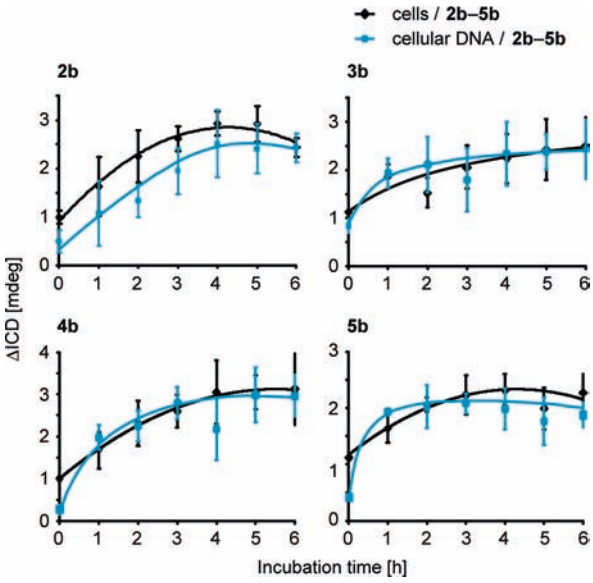

Figure 7. Induced CD (ΔICD = Ellipticity (λ = 335 nm) − Ellipticity (λ = 305 nm)) after exposure of cells to the *seco*-drugs **2b**−**5b**. All data points reflect mean values ± s.d.

branes of the cancer cells very quickly to allow an interaction with the cellular DNA. Thereby, the drugs form covalent adducts with the DNA after a preliminary noncovalent binding.

All measurements were carried out at least three times and the ΔICD values, that is the differences in the CD-intensities at the extrema at $\lambda = 305$ nm and $\lambda = 335$ nm, were calculated for each point in time (Figure 7). A comparison of the time-dependent changes in the ΔICD reveals that all *seco*-drugs display similar kinetics for uptake into the cells and for the interaction with the cellular DNA.

Discussion

Understanding the mode of action of drugs and knowing structure activity relationships is very important for a sophisticated and safe administration of drugs and for drug development. We report here on the use of CD spectroscopy for the study of the cellular uptake and the mechanism of action of several new anticancer drugs. A characteristic induced CD with a minimum at $\lambda = 305$ nm and a maximum at $\lambda = 335$ nm was observed for mixtures of the *seco*-drugs **2b**−**5b** and synthetic DNA oligonucleotides (Figure 4). An identical shape and location of the induced CD was found for suspensions of live cells pretreated with the same *seco*-drugs (Figure 5) and for the DNA isolated out of these cells (Figure 6). Additionally, a CD signal characteristic for the *seco*-forms of the anticancer agents was observed for cells incubated with the more toxic compounds **3b** and **5b**.

Very short incubations (<1 min) of the cells with the *seco*-drugs followed by immediate washing steps ($t = 0$ h) resulted in a strong induced CD for the cells but only in a small induced CD for the isolated DNA (Figures 5−7). In contrast, after longer incubation times, the induced CD of the cell suspensions and the isolated DNA was almost identical. From these results, it can be deduced that the *seco*-drugs and their corresponding in situ formed drugs with a spirocyclopropyl moiety penetrate the cell membrane very quickly and that the drugs immediately form a noncovalent complex with the DNA. This noncovalent bonding is followed by a slower alkylation to give covalent drug−DNA complexes, which can be isolated from the cells.

Cells incubated with the *seco*-drugs **3b** and **5b** show an additional maximum at $\lambda = 390$ nm (Figure 5) that is not observed for the DNA isolated from the treated cells (Figure 6). Furthermore, the more toxic the *seco*-drug, the stronger is the signal and the longer is its persistence, i.e.: **5b** > **3b** ≫ **2b** ∼ **4b**. The CD-signal at $\lambda = 390$ nm is caused by the *seco*-forms **2b**−**5b** and once the *seco*-drugs have cyclized to give the corresponding drugs, this signal is no longer observed. We therefore assume that the more toxic *seco*-drugs **3b/5b** are stabilized inside the cells by interaction with specific regions of the DNA. Additionally, their corresponding drugs **3c/5c** are more stable toward nucleophilic attack. As a consequence, the inactivation of the active drugs by hydrolysis is reduced compared to the less toxic drugs of **2b/4b** so that the overall DNA alkylation using the more toxic compounds **3b/5b** is increased, although their reaction rate is lower as compared to **2b/4b**. This is in agreement with previous findings[20] and with our investigations of the hydrolytic stability of **2b**−**5b** in solution using HPLC−MS. Here, more toxic drugs like **3c/5c**, containing an unsubstituted cyclopropyl moiety, were more stable against hydrolysis than less toxic drugs with a methyl substituent at the cyclopropyl unit, such as **2c/4c**.

Experimental Section

Materials. *seco*-drugs **2b**−**5b** were synthesized according to previously published procedures.[10c,d] The synthetic double-stranded DNA oligomer 5′-d(CGGTCAATTAGTCGC)-3′ · 3′-d(GCCAGTTAATCAGCG)-5′ was purchased from IBA (Göttingen, Germany) as aqueous solution (0.1 mm) of the respective sodium salt.

CD-Spectroscopy. All CD-spectra were recorded using a Jasco J-810 spectropolarimeter at 25 °C. Data mode: CD and Abs, bandwidth 1.0 nm, response: 1 s, sensitivity: 100 mdeg, step resolution: 0.1 nm, scan speed: 100 nm min^{-1}, four accumulations.

Cell Culture. Human promyelocytic leukemia cells of line HL-60 were kindly provided by the Department of Cellular and Molecular Immunology, University of Göttingen (Göttingen, Germany). Cells were maintained as exponentially growing cultures at 37 °C and 5.0% CO_2 in air in a culture medium consisting of RPMI 1640 (Biochrom) supplemented with 10% fetal calf serum, 44 mM $NaHCO_3$ (Biochrom) and 2 mM L-Glutamine (Invitrogen).

CD Spectroscopy of *seco*-Drugs 2b−5b. Stock solutions of the *seco*-drugs **2b**−**5b** were prepared in DMSO. All measurements were performed using a concentration of 58 μM of the respective *seco*-drug in PBS (pH 7.4). The CD-spectra were recorded using a 1 cm quartz cuvette (Hellma).

CD Spectroscopy of a Mixture of DNA Oligonucleotides and *seco*-Drugs 2b−5b. Double-stranded DNA oligonucleotides (5′-d(CGGTCAATTAGTCGC)-3′ · 3′-d(GCCAGTTAATCAGCG)-5′ were used as aqueous solutions of the corresponding sodium salts. Stock solutions of the *seco*-drugs were prepared in DMSO. All measurements were performed in PBS (pH 7.0) with concentrations of 6 μM of DNA or 6 μM of DNA and 6 μM of the respective *seco*-drug under investigation. The CD-spectra were recorded using a 1 cm quartz cuvette (Hellma).

Incubation of Cells with *seco*-Drugs 2b−5b followed by CD Spectroscopy. Cells were washed with serum-free cell culture medium (UltraCulture medium, Lonza). A suspension of cells in UltraCulture medium was pipetted in 6 multiwell plates at concentrations of 3×10^6 cells per mL and well. Cells were then incubated in UltraCulture medium (2 mL) containing 1% DMSO (control) or 1% of a freshly prepared solution of the respective *seco*-drug in DMSO.

(20) (a) Tichenor, M. S.; MacMillan, K. S.; Stover, J. S.; Wolkenberg, S. E.; Pavani, M. G.; Zanella, L.; Zaid, A. N.; Spalluto, G.; Rayl, T. J.; Hwang, I.; Baraldi, P. G.; Boger, D. L. *J. Am. Chem. Soc.* **2007**, *129*, 14092–14099. (b) Parrish, J. P.; Hughes, T. V.; Hwang, I.; Boger, D. L. *J. Am. Chem. Soc.* **2004**, *126*, 80–81. (c) Boger, D. L.; Yun, W. *J. Am. Chem. Soc.* **1994**, *116*, 5523–5524.

seco-drug concentrations were 58 μM for *seco*-drugs **2b**−**4b** and 41 μM for *seco*-drug **5b**. Directly after starting the incubation or after different times of exposure to the drugs, the test substance was removed and the cells were washed 3× with PBS (pH 7.4), resuspended in 70 μL PBS, filled into the chamber of a 0.1 mm thin layer quartz cuvette (Hellma), and the CD- and absorption spectra were recorded. All kinetics were measured three times.

Incubation of Cells with *seco*-Drugs 2b−5b followed by DNA Isolation and CD Spectroscopy. Cells were washed with serum-free cell culture medium (UltraCulture medium, Lonza). A suspension of cells in UltraCulture medium was pipetted in 6 multiwell plates at concentrations of 5×10^6 cells per mL and well. Cells were then incubated in UltraCulture medium (2 mL) containing 1% DMSO (control) or 1% of a freshly prepared solution of the respective *seco*-drug in DMSO. *seco*-drug concentrations used were 97 μm for *seco*-drug **2b**, 68 μM for *seco*-drugs **3b** and **4b** and 48 μM for *seco*-drug **5b**. Directly after starting the incubation or after different times of exposure to the *seco*-drugs, the test substance was removed, the cells were washed 3× with PBS (pH 7.4) and then resuspended in 200 μL PBS. An 8-μL portion of the cell suspension was added to a mixture of 192 μL PBS (pH 7.4) and 50 μL Trypan blue (Biochrom, 0.5% (w/v) in physiological saline) and the fraction of dead cells was macroscopically determined. The remaining cell suspension was used to purify the cellular DNA using a QIAamp DNA Mini Kit (Qiagen) and additional RNase A (Qiagen). DNA was eluted with 200 μL bidestilled water and the concentration was adjusted to 23 μg/mL (UV-absorption: 0.46) for *seco*-drugs **2b** and **3b**, or 30 μg/mL (UV-absorption: 0.60) for *seco*-drugs **4b** and **5b**. The CD-spectra were recorded using a 1-cm quartz cuvette. All kinetics were measured four times.

Conclusions

We have exemplarily demonstrated, for several potential anticancer drugs based on the natural antibiotic CC-1065, that time-dependent CD spectroscopy of suspended live cells after exposure to the drugs gives information about the cellular uptake of these drugs as well as of their interaction with biopolymers such as DNA inside the cells. Furthermore, analysis of the CD spectra even allow explanation of different cytotoxicities of similar drugs. The anticancer agents rapidly penetrate the cell membrane and bind to the cellular DNA as their intracellular target. The formation of a reversible noncovalent complex with the DNA is followed by a covalent binding of the drug to the DNA. Thereby, the more toxic *seco*-drugs and their corresponding drugs show a higher stability and a lower alkylation rate.

The studies have led to a deeper understanding of the complex mode of action of novel members of a class of potential anticancer agents that are of wide interest in the scientific community. In addition, the method described is supposed to allow the investigation of the mode of action of bioactive compounds in general provided that the interaction of the respective compound and the cellular target molecules causes a significantly induced CD signal.

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