Antitumor Agents

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The Two Faces of Potent Antitumor Duocarmycin-Based Drugs: A Structural Dissection Reveals Disparate Motifs for DNA versus Aldehyde Dehydrogenase 1 Affinity**

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Duocarmycin SA (**1**) and (+)-CC-1065 (**2**) (Scheme 1 a) belong to a class of well-known and potent antitumor natural products that were first isolated from *Streptomyces*.[1] Different analogues progressed into clinical phase I and II, but their hematologic toxicity led to severe side effects and termination of treatment.[1c,2] One promising strategy to overcome these limiting effects is the antibody-directed enzyme prodrug therapy (ADEPT), which combines the use of "nontoxic" prodrugs and antibody-conjugated enzymes that control drug release.[3] Excellent results were achieved with prodrug **3** (Scheme 1 b), which contains a seco-CBI[4] analogue of duocarmycin (CBI = cyclopropabenzindole) connected to a galactose moiety.[5] A β-D-galactosidase-mediated deglycosylation of prodrug **3** gives the corresponding seco-drug **4** with a phenolic hydroxy group, which is further processed in situ by a Winstein cyclization to afford the highly cytotoxic CBI drug **5**.[5]

Duocarmycins act through sequence-specific DNA alkylation and interstrand crosslinking.[1a,4a,6] This is achieved by a dual mode of action where the DNA-binding subunit (Scheme 1 c) first incorporates noncovalently into AT-rich sites of the minor groove followed by an alkylation at the N3-position of 3′adenine with the electrophilic cyclopropane.[1a,4a,6] The DNA-binding subunit is crucial for DNA recognition and alkylation, since complete replacement of the indole by for example, tert-butoxycarbonyl (Boc),[4a,7] acetyl,[7a] or various mono- and bicycles,[8] significantly decreased the efficiency of DNA alkylation. Although significantly less potent compared to the natural product (+)-CC-1065, these analogues still exhibited nanomolar IC_{50} values.[4a,7–8]

Recently, a new class of bifunctional analogues (Scheme 1 c), which are built up by two symmetrically linked CBI alkylation subunits (**7**) revealed a lack of DNA affinity in in vitro binding studies.[9] Still these analogues are highly cytotoxic compounds with IC_{50} values of as low as 110 fM based on the human tumor colony forming ability assay (HTCFA), which reflects the proliferation capacity of single cells. These results emphasized that other targets than DNA could be involved in binding.[9] In a previous study we utilized an activity-based protein profiling (ABPP) approach with an alkynylated seco-drug probe **8** (Scheme 1 c).[10] This probe molecule was incubated with intact A549 lung cancer cells and revealed aldehyde dehydrogenase 1 (ALDH1A1) as a prominent non-DNA target of duocarmycin derivatives.[10a] ALDH1A1 is responsible for the oxidative formation of retinoic acid and thus influences gene expression and differentiation.[11] High expression in several tumors such as pancreatic,[12] breast,[13] and lung cancers[14] correlates with increased proliferation and results for the latter two in an aggressive phenotype that tends to end up in a poor prognosis. In cancer cells ALDH1A1 influences diverse cellular pathways that are not fully elucidated yet. Thus it is an attractive target for in-depth characterization. However, the individual structural elements of duocarmycin analogues that contribute solely to ALDH1A1 inhibition remained unknown to date.

We here chemically dissected the duocarmycin scaffold and identified structural elements that are important for ALDH1A1 binding and inhibition. For these studies, we first removed the DNA-binding subunit of a duocarmycin analogue (such as **5**) and synthesized pure CBI alkylation subunits containing an alkyne moiety for fluorescent tagging by using click chemistry.[15] Furthermore, we synthesized an alkynylated version of the bifunctional seco-drug **11** (Scheme 2). Application of these probes in A549 lung cancer cells successfully revealed a high selectivity and specificity for ALDH1A1. Moreover, it was possible to determine the efficiency of DNA and/or protein alkylation in the native environment with different analogues by fluorescent imaging. Although the novel CBI compounds did not bind to DNA anymore, they still exhibited remarkable cytotoxicity. In addition, the essential role of ALDH1A1 for

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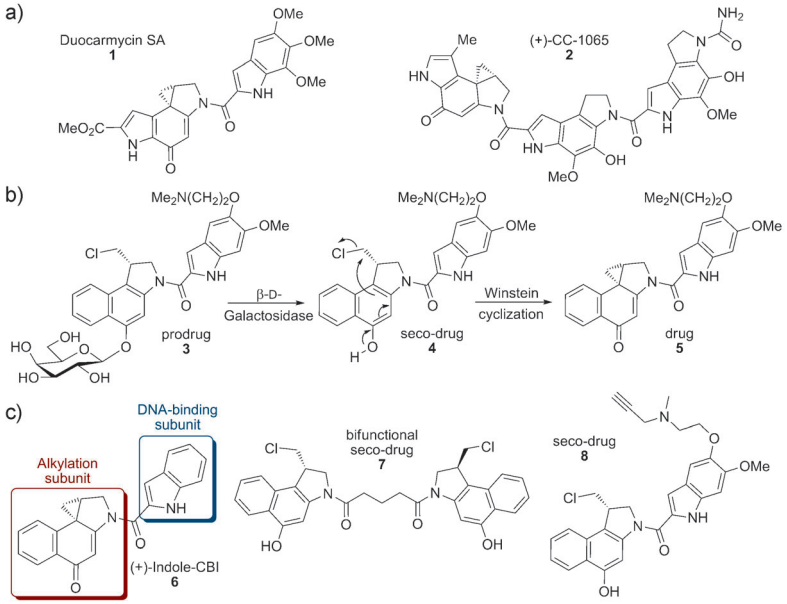


Scheme 1. a) Structures of duocarmycin SA and (+)-CC-1065. b) The ADEPT strategy illustrated by the activation of a CBI alkylation subunit (CBI = cyclopropabenzindole). The transformation of the glycosylated prodrug is executed by β-D-galactosidase and leads to the corresponding drug via the intermediate seco-drug. c) Dissection of an indole-CBI scaffold into an alkylation subunit (AS) and a DNA-binding subunit (DBS) and further structures of bifunctional seco-drug **7** and the clickable seco-drug **8**.

Scheme 2). To control for any adverse effects of the alkyne handle, we designed seco-drug **10**.

We first determined effects of the novel seco-drugs **9–11** and the established DNA-binding seco-drug **8**[10a] on cell proliferation in the HTCFA assay, which is based on the capability of cells to form new clones after treatment with small molecules (Figure 1). This assay reflects the long-term cytotoxicity after ten days of initial exposure. While the seco-drug **8** exhibited the highest cytotoxicity with an IC_{50} value of 14 pM, the novel compounds **9, 10**, and **11** revealed higher IC_{50} values of 5.3 nM, 17.6 nM, and 0.11 nM, respectively. Still all novel compounds exhibited potent cytotoxicity in the low-nM or high-pM range, thus raising the question of their corresponding targets.

Previous ABPP studies on A549 lung cancer cells revealed that seco-drug **8** was not only characterized by its DNA-alkylating properties but in addition by a covalent binding to cysteine residues 456 and 464 of ALDH1A1.[10a] We therefore assumed that removal of the DNA-recognition unit in seco-drug **8** would result in an unchanged or even improved ALDH1A1 affinity that allows to evaluate the contribution

cellular viability was confirmed by siRNA knockdown experiments. The results obtained here do not only give a structural explanation for the dual target preferences of duocarmycin analogues but also provide methods for focused ALDH1A1 studies that may further enable the characterization of this important cancer-associated enzyme and guide the design of novel duocarmycin-based drugs and prodrugs.

To determine the structural moiety that is responsible for ALDH1A1 binding and inhibition we designed several analogues that solely contain the CBI motif (lacking the DNA-binding indole), which is important for alkylation. We synthesized seco-drugs **9–11** starting from enantiomeric pure Boc-protected seco-CBI **12** as shown in Scheme 2 (detailed synthesis in the Supporting Information). A HCl-mediated deprotection of **12** was subsequently followed by an EDC[16]-activated coupling with the alkynoic and alkanoic acids **13** and **14** and the diacid **15** (synthesis presented in Supplementary

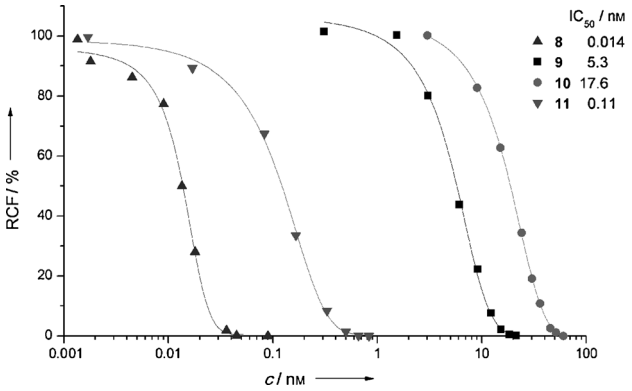
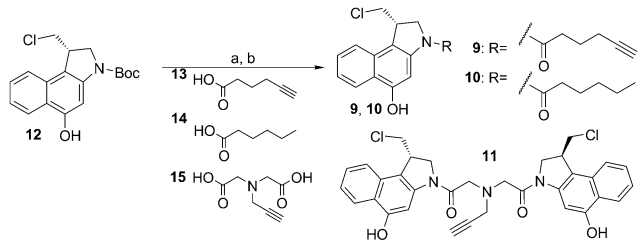


Figure 1. Plot of the relative clone-forming rate (RCF) against different concentrations *c* of compounds **8–11** to determine the long-term cytotoxicity in A549 cells (HTCFA).



Scheme 2. Synthesis of clickable seco-drug **9**, control compound **10**, and dimeric seco-drug **11** without DNA-binding subunit. a) 4 N HCl/ethyl acetate, RT, 3 h; b) **13–15**, EDC·HCl, DMF, RT, 15–18 h, **9**: 67%, **10**: 29%, **11**: 31%.

of this unit to biological potency. To validate this hypothesis compounds **9–11** as well as seco-drug **8** were applied to ABPP studies and incubated in situ with A549 cells for four hours. After cell lysis, the labeled proteins were tagged with rhodamine azide by using click chemistry and separated by gel electrophoresis. Only one prominent fluorescent band was detected for all probes (with the exception of control compound **10** lacking the alkyne tag). Based on our previous mass-spectrometric analysis with probe **8** we were able to assign this protein as aldehyde dehydrogenase 1 (Figure 2a, red arrow). Furthermore, the identity was independently confirmed by Western blot analysis (Figure 2b).

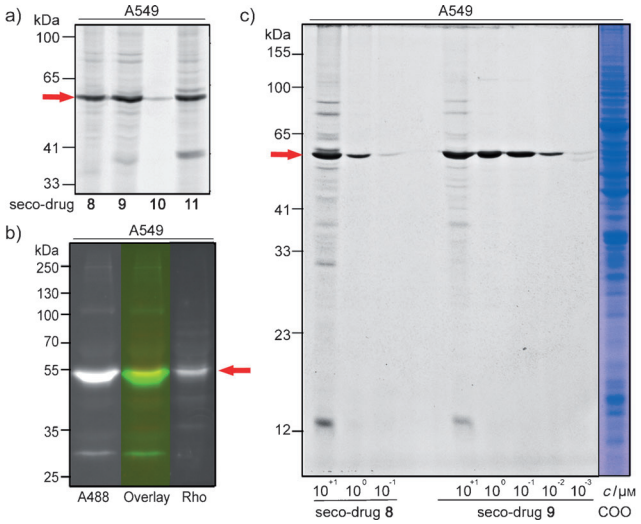
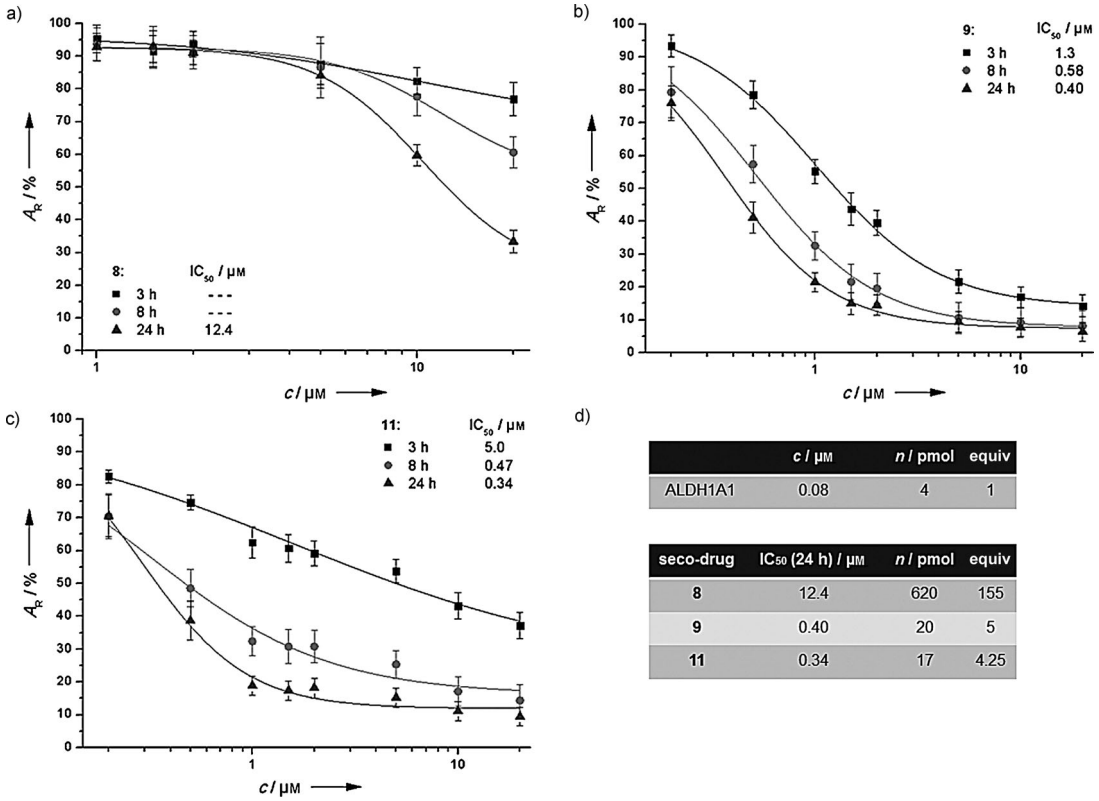


Figure 2. a) Comparison of in situ labeling pattern with seco-drugs **8**–**11** (10 µM, 4 h) of A549 cells on an SDS-PAGE gel. The aldehyde dehydrogenase is marked with a red arrow. b) Western blot of A549 cells labeled with compound **9** (10 µM, 4 h; A488 = Alexa 488 + ALDH1A1 antibody, Rho = rhodamine). c) SDS-PAGE gel of A549 cells labeled in situ (4 h) with decreasing concentrations of compounds **8** (+ DBS) and **9** (-DBS). In-gel fluorescence scanning was used for the images of the gels in (a–c), and the lane containing 1 nM **9** was stained with coomassie blue (COO).

To further compare the affinity for the aldehyde dehydrogenase 1, A549 cells were incubated with serial dilutions of DNA-binding seco-drug **8** and seco-drug **9** without DNA-binding unit for 4 h (Figure 2c). While compound **8** showed a visible band down to a concentration of 100 nM, labeling with compound **9** could be achieved down to a concentration of even 1 nM. These results suggest that removal of the DNA binding motif indeed resulted in compounds with a significantly increased affinity for ALDH1A1. Moreover, no labeling of other proteinogenic targets could be observed at concentrations below 1 µM (Figure 2c).

In a next step we compared the in vitro inhibitory efficacy of DNA-binding seco-drug **8** with seco-drugs **9** and **11** against recombinantly expressed and purified ALDH1A1 by using an established ALDH1A1 activity assay with propanal as a substrate.[10a] Since ALDH1A1 inhibition occurs rather slow and through a covalent mode of action, the enzyme activity was measured after preincubation for 3, 8, and 24 h with different compound concentrations to observe the corresponding time-dependent IC_{50} shifts. Of note, the minimal observable IC_{50} values are limited by the concentration of recombinant enzyme used in the in vitro assay of 0.08 µM. As expected for irreversible inhibitors, the IC_{50} values decreased with increasing incubation time to 0.40 µM and 0.34 µM for compound **9** and **11** after 24 h (Figure 3b,c), while DNA-binding seco-drug **8** (Figure 3a) showed a rather weak inhibition with an IC_{50} value of 12.4 µM. Considering the above-mentioned limitation of the in vitro assay, these values correspond to only 5 and 4.25 molecules of compounds **9** and **11** per molecule of enzyme, which is already sufficient to inhibit 50% of the ALDH1A1 activity after 24 h (Figure 3d). These results are in line with the above ABPP study and further



	c / µM	n / pmol	equiv
ALDH1A1	0.08	4	1

seco-drug	IC_{50} (24 h) / µM	n / pmol	equiv
8	12.4	620	155
9	0.40	20	5
11	0.34	17	4.25

Figure 3. a–c) Remaining enzyme activity A_R of ALDH1A1 after inhibition with CBI derivatives **8**, **9**, and **11** for 3, 8, and 24 h. d) Calculation of the equivalents of seco-drugs **8**, **9**, and **11** needed to inhibit 50% of ALDH1A1 activity after 24 h of incubation.

demonstrate an improved affinity of ALDH1A1 for those compounds that lack the DNA binding motif.

So far we focused our studies on the binding and inhibition of ALDH1A1 and obtained an increased affinity for those compounds that lack the DNA-recognition unit. To investigate if this increased affinity for ALDH1A1 correlates with a decrease of DNA binding, we subsequently utilized whole-cell imaging in combination with fluorescent immunostaining for selected probe molecules. A549 cells were grown on coverslips and incubated in situ with various concentrations of **7, 8**, **9, 10**, and **11** for 2–4 h. Subsequently the cells were fixed with paraformaldehyde, permeabilized with saponine, and blocked with bovine serum albumine (BSA). The primary ALDH1A1 antibody was incubated over night at 4 °C and tagged with a secondary antibody containing Atto 488 as fluorophore. Thereafter the cells were treated with rhodamine azide under click-chemistry conditions, and finally the DNA was stained with 4′,6-diamidino-2-phenylindole (DAPI). As expected, compound **8** showed a high affinity for DNA (Figure 4a and Supplementary Figures 1a and 3a), since intense fluorescence signals could be observed in the nucleus and weaker protein fluorescence signals in the cytosol (Supplementary Figure 2). Contrary, seco-drugs **9** and **11** exhibited negligible DNA labeling as shown by the very weak fluorescence of the nucleus, thus indicating that in a native environment these seco-drugs likely do not exert their biological activity through DNA alkylation (Figure 4b,c and Supplementary Figures 1b,c and 3b,d). Importantly, strong fluorescence signals were observed in the cytosol, thereby suggesting binding of compounds **9** and **11** to a protein target. This signal was even observed at concentrations as low as 10 nM of compound **9**, thus emphasizing a high binding affinity (Supplementary Figure 4). Immunostaining of the cells with an ALDH1A1-specific antibody revealed a colabeling in the cytosol between the probe (**9**) signal and ALDH1A1 immunofluorescence. Control compounds **7** and **10** lacking the alkyne for click chemistry showed no fluorescent signal (Supplementary Figure 3c,e).

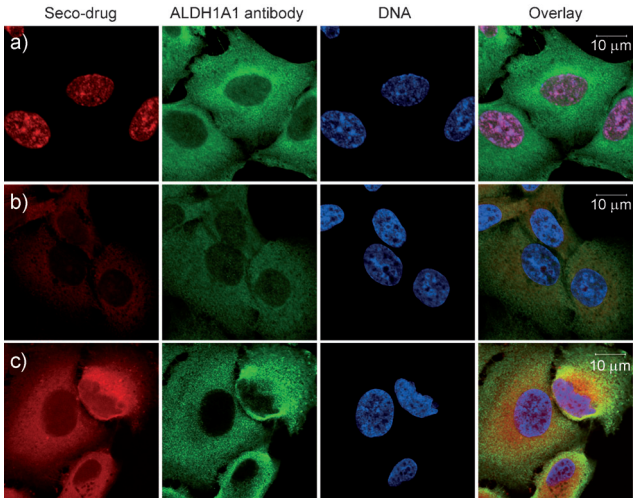


Figure 4. Cell imaging of A549 cells with different duocarmycin derivatives and immunostaining with an ALDH1A1 antibody. The images were recorded with a confocal microscope. The primary ALDH1A1 antibody was labeled with a fluorescent secondary antibody (Atto 488, green). Rhodamine azide (red) was attached by using click chemistry to the alkyne-containing duocarmycin probes. The DNA is stained with DAPI (blue). The corresponding negative controls are presented in Supplementary Figure 3. Black-and-white pictures for a better comparison of the fluorescence signal are presented in Supplementary Figure 1. a) With DNA binder (1 μM seco-drug **8**, 2 h incubation). b) With non-DNA binder (1 μM seco-drug **9**, 2 h incubation). c) Bifunctional CBI (1 μM seco-drug **11**, 4 h incubation).

ALDH1A1 has been described as an essential enzyme for cell viability in A549 cells.[17] To establish a connection between the cytotoxicity of seco-drugs lacking the DNA-binding subunit and ALDH1A1, we utilized siRNA to knock down the expression and to investigate the corresponding effects on cell proliferation. Therefore A549 cells were transfected with four different ALDH1A1-specific siRNAs and were monitored by Western blot for six days (Supplementary Figure 5). Three days after transfection all siRNAs provided a high knock-down efficiency with 80% reduction of protein expression compared to the scrambled control siRNA (scr). To determine the effect on proliferation, the increase of cell density was monitored between the third and sixth day after transfection of A549 cells with siRNAs 1, 3, and scr. The results (Supplementary Figure 6a) demonstrate a significantly reduced proliferation after three days of growth to approximately 64% and 57.5% of siRNA 1 and 3 in relation to scr (Western blot, Supplementary Figure 6b). Furthermore, incubation of siRNA-scr-treated A549 cells with seco-drugs **9** and **11** over the same period of time exhibited a comparable reduction of proliferation at concentrations between 10–20 nM and around 5 nM, respectively (Supplementary Figure 6c,d). These results confirm that ALDH1A1 is an essential enzyme for proliferation in A549 cells and show that treatment of cells with low nM concentrations of **9** and **11** reveal phenotypes comparable to ALDH1A1 siRNA knockdowns.

In conclusion, we utilized a broad range of methodologies to dissect a duocarmycin-inspired scaffold and investigated the structural basis for its recently discovered ALDH1A1 affinity. There is no doubt that duocarmycin analogues with an intact indole moiety bind DNA and possibly exert a large part of their bioactivity through this pathway. However, analogues lacking this moiety lose their DNA affinity and gain specificity for ALDH1A1. Although reduced in potency, CBI compounds **9**, **10**, and **11** lacking the indole motif still exhibit remarkable cytotoxicities that are matched by the essential role of ALDH1A1 in lung cancer cell proliferation. Of note, selective ALDH1A1 inhibitors are of high interest in the targeting of cancer stem cells.[18] We would like to stress that our study focused on covalent protein interaction partners of CBI. Reversible binding to other potential protein or nonproteinogenic targets cannot be excluded and will be investigated in future studies. In addition, similar investigations should be performed with the CPI unit, which is found in duocarmycin (**1**) and CC-1065 (**2**).

Note added in proof: Recently it has been doubted that ALDH1A1 is an important target for duocarmycin analogues lacking the DNA-binding indole subunit. However, we are convinced that the investigations described here strongly support this interaction.[19]

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