Drug Development

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Duocarmycin Analogues Target Aldehyde Dehydrogenase 1 in Lung Cancer Cells**

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Duocarmycin SA (**1**, Figure 1a), an antibiotic metabolite isolated from *Streptomyces* DO-113, has received considerable attention as it is one of the most potent antitumor agents known to date and has IC_{50} values of about 10 pM against different cancer cell lines.[1,2] In spite of this potency, a direct medical application for chemotherapy is limited as a result of pronounced myelotoxicity.[3,4] The challenge to eliminate this undesired toxicity was addressed by the introduction of an efficient prodrug concept, in which the reactive scaffold, the cyclopropyl moiety, is formed in situ by an intramolecular cyclization from seco-drug **2** or similar compounds (Figure 1a).[1,5,6] Thus, the cyclopropyl group is formed by a reaction between the chloromethyl group and an arene moiety that contains a free phenolic hydroxy group. This hydroxy group is generated by a β-D-galactosidase-mediated removal of D-galactose from the prodrug **3**. The value of this approach was demonstrated by an efficient galactosidase-catalyzed conversion of **3** with low cytotoxicity into the corresponding duocarmycin analog **2**, which has high cytotoxicity.[1] As galactosidase can be linked to cancer cell specific antibodies, prodrug **3** becomes activated only at tumor sites, which provides a selective targeting strategy called antibody-directed enzyme prodrug therapy (ADEPT).[1,7]

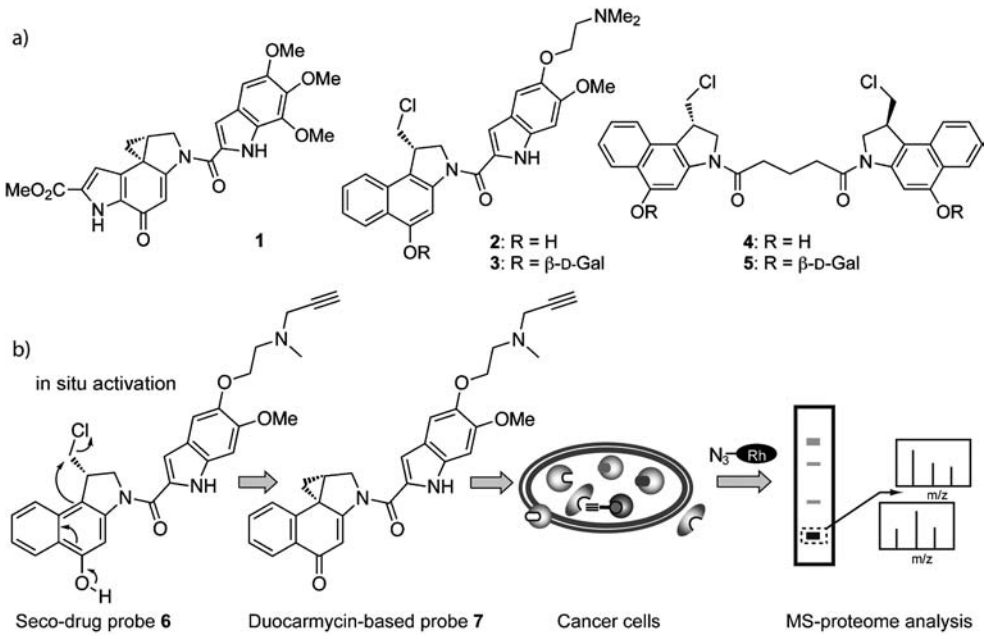

Figure 1. a) Structures of duocarmycin SA (**1**), seco-drugs **2** and **4**, as well as glycosidic prodrugs **3** and **5**. b) In situ activation of the seco-drug probe **6** leads to the duocarmycin-based species **7**, which covalently binds to aldehyde dehydrogenase 1 in living cancer cells. Protein identification is achieved by click chemistry (CC), SDS separation, and MS analysis.

Previous studies revealed that duocarmycins and similar compounds exert their potent antitumor properties by a very fast, noncovalent incorporation into the minor groove of double-stranded DNA, and subsequent fixation through a sequence-selective adenine *N*-3 alkylation to induce apoptosis.[8] Furthermore, the unusually poor reactivity of duocarmycin analogs with conventional nucleophiles, such as glutathione, suggests that DNA is the dedicated target of these natural products.[9] However, this theory was brought into question by the recent finding that the bifunctional duocarmycin-derived seco-drug **4**, which does not contain the indole-type DNA-binding unit, has an even higher cytotoxicity ($IC_{50} = 110$ fM) relative to **2**. As it has been shown that **4**

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does not react with DNA, one could assume that additional proteomic targets may have been overlooked so far (Figure 1a).[10] To address this question, we applied a chemical proteomic strategy, termed activity based protein profiling (ABPP),[11–13] which revealed duocarmycin based seco-drug **6** and its bifunctional analog **4** as selective and potent inhibitors of aldehyde dehydrogenase 1 (ALDH1A1) in lung cancer A549 cell lines.

One prerequisite for ABPP target discovery is the chemical design of a probe scaffold that contains an alkyne handle, which serves as a benign tag for modification with functionalized azides by Huisgen-Sharpless-Meldal click chemistry (CC) reaction, to visualize, enrich, and identify bound targets (Figure 1b).[14–16] The synthetic strategy is detailed in Scheme S1 in the Supporting Information. In brief, **6** was obtained by 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC)-activated coupling of the seco-chlorobenzindole derivative **8** and the DNA-binding **10**, which bears the alkyne group for labeling purposes. Compound **10** was prepared through nucleophilic substitution of the chloride in **9** (Scheme S1 in the Supporting Information).

Another important prerequisite of ABPP concerns the biological potency of the probe molecule, which should not be significantly lower relative to the parent compound. A direct comparison of the individual IC_{50} values with the human tumor colony forming ability assay (HTCFA) revealed that **6** was about as potent ($IC_{50} = 14$ pM) as the unmodified compound **2** ($IC_{50} = 9$ pM), which indicates no adverse effects from the attached alkyne (Figure S1 in the Supporting Information). As the HTCFA assay reflects long term cytotoxicity, we additionally obtained acute cytotoxicity within 24 h of incubation by the MTT test, which revealed IC_{50} values of about 0.2 nM for **2**, **4**, and **6** (Figure S2 in the Supporting Information). To analyze the cellular targets of **6** in cancer cells we incubated **6** with living A549 lung cancer cells at various concentrations for 4 h. Subsequently, the cells were lyzed and the proteome was treated with rhodamine azide under CC conditions. Fluorescent SDS-PAGE analysis of the labeled proteome revealed only a single strong protein band at a molecular weight of about 60 kDa (Figure 2a). Interestingly, this protein band appears only as a weak signal in cellular lysates (in vitro) relative to living, intact cells (in situ), which emphasizes that the corresponding protein is significantly inactivated after cell rupture. Intense fluorescent labeling could be observed down to a probe concentration of 100 nM, which emphasizes a specific protein recognition and high-affinity binding (Figure 2b).

To identify this protein, we applied a quantitative enrichment procedure by which living cells were incubated with **6**, lyzed, and treated with a trifunctional rhodamine-biotin-azide linker in a click reaction. The trifunctional linker allows the selective enrichment of probe-labeled proteins by using avidin beads. Subsequent fluorescent SDS-PAGE revealed a strong 60 kDa band that was isolated and subjected to mass spectrometry (MS/MS analysis). The peptide fragments that were obtained were subjected to the SEQUEST search algorithm and ALDH1A1 was identified as a strong hit (Table S1 in the Supporting Information). To independently confirm the results of the MS, we labeled commercially

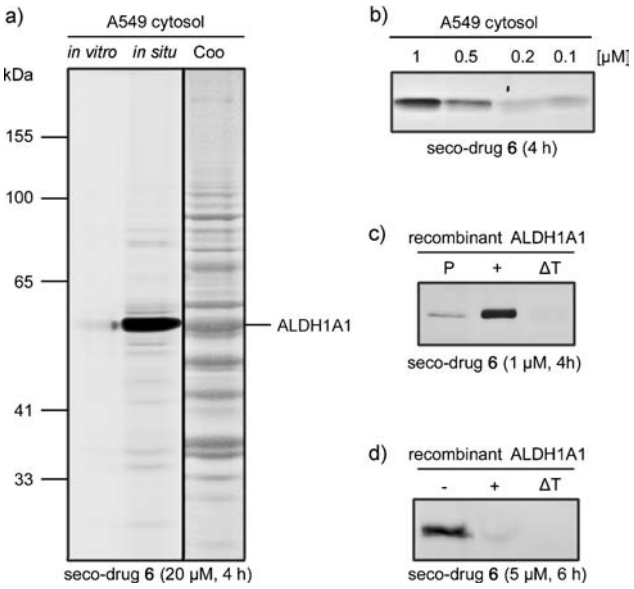

Figure 2. a) Results of labeling by fluorescent SDS analysis. Coo = Coomassie staining. b) Serial dilutions of **6**. c) Recombinant ALDH1A1 is labeled by the probe only in its native form. P = native Proteom; + = recombinant ALDHA1; ΔT = heat denaturation prior to labeling. d) Competitive labeling of recombinant ALDH1A1 by **6** and a 40-fold excess of dimeric **4**. − = incubation with DMSO prior to labeling with **6**; + = incubation with **4** prior to labeling with **6**; ΔT = heat denaturation prior to labeling).

available ALDH1A1 with **6**. Subsequent fluorescent SDS-PAGE analysis clearly shows an intense 60 kDa band in the case of the native, folded protein, which disappears after heat denaturation of the protein and, therefore, suggests a specific probe binding (Figure 2c).

Labeling of ALDH1A1 represents an intriguing result, as this enzyme plays crucial roles in cancer-cell proliferation, as well as in the inactivation of several cancer drugs, such as cyclophosphamide and oxaphosphorines.[17,18] Lung cancer cells have been reported to express elevated levels of ALDH1A1, in which the enzyme is responsible for oxidizing intracellular aldehydes (including those derived from tobacco smoke) and contributes to the conversion of retinol to retinoic acid in early cancer stem cell differentiation.[19,20] This broad substrate tolerance correlates with a huge diversity of functions that have probably not all been discovered yet. Moreover, several knockdown studies with ALDH1A1-specific siRNA showed that this enzyme is important for cell proliferation and survival.[21–23] Therefore, selective inhibition of ALDH1A1 by duocarmycin analogs, such as **2**, seems to be an additional route by which these molecules are active. Moreover, for **4**, which lacks DNA-binding properties and has an increased cytotoxicity, this might be the preferred mode of action.[10]

To investigate this hypothesis, we first analyzed the binding of **6** to recombinant ALDH1A1 by MS/MS sequencing. Two different peptide fragments were identified that contain probe-bound cysteine residues (Cys456 and Cys464), which indicates that two molecules of **6** bind per enzyme subunit (Figure 3a, see also Figure S3 and Table S2 in the Supporting Information). Previous crystallographic data

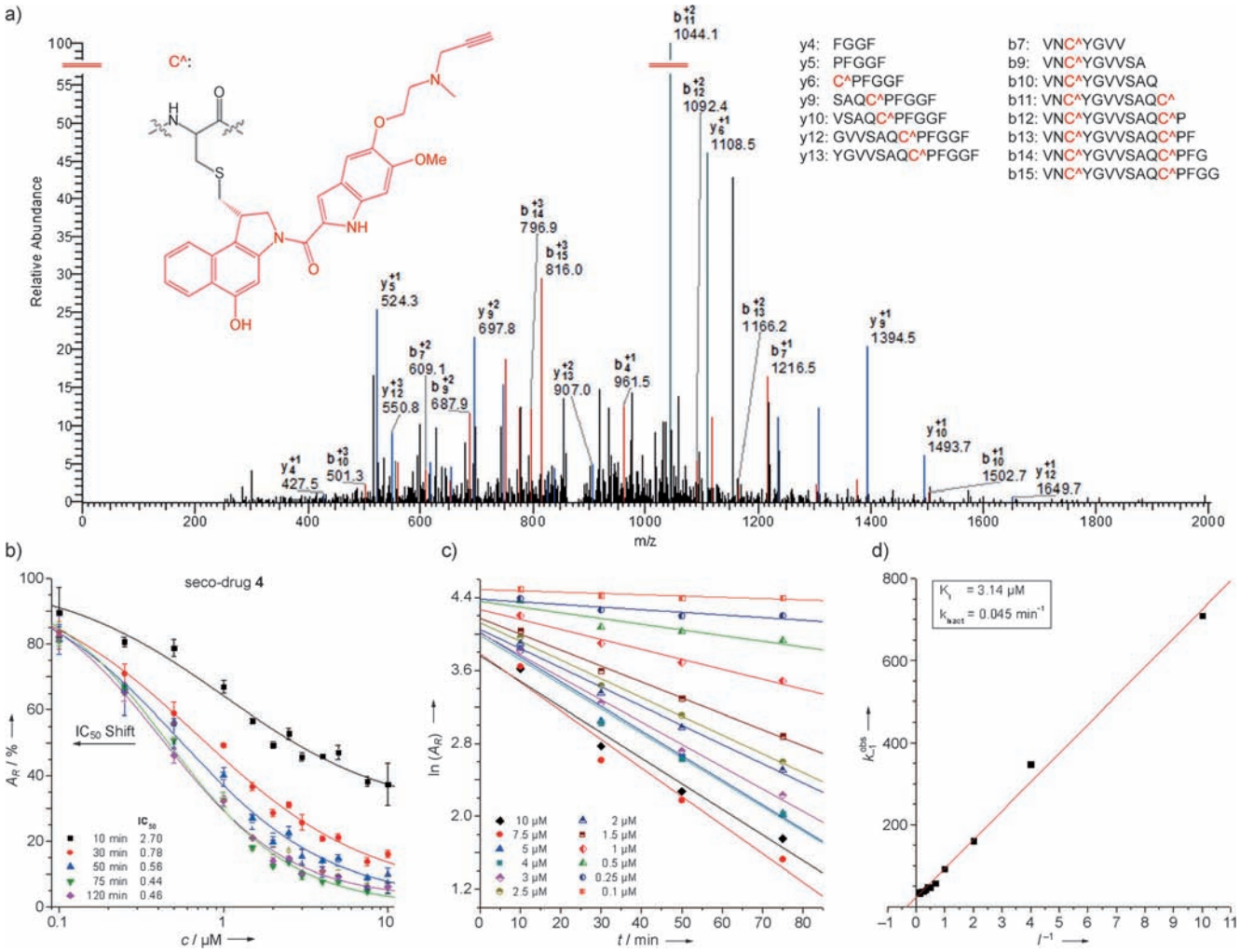

Figure 3. ALDH1A1 inhibition and mode of binding. a) MS/MS sequencing shows the binding of **6** to Cys456 and Cys464. Figure S3 in the Supporting Information shows the corresponding data for a different peptide fragment. b) Time dependent IC_{50} shift of **4** measured with propanal. c) Linear fit of the natural log of the remaining enzyme activity A_R against the pre-incubation time and determination of k_{obs} from the slopes. d) Double-reciprocal plot of k_{obs} against inhibitor concentration to determine k_{inact} and K_I. Inactivation plots for **6** are shown in Figure S6 in the Supporting Information. A summary of all K_I and k_{inact} values is summarized in Table S3 in the Supporting Information.

shows that these two cysteine residues are located at a remote position with respect to the enzyme active site.[24] Pre-incubation of ALDH1A1 with **4** and subsequent addition of **6** abolished labeling, which emphasizes that both compounds compete for the same binding site (Figure 2 d). Although the peptide modified by **4** could not be detected by various MS/MS experiments as a result of insufficient ionization, it is possible that **4** bridges both remote cysteine residues by a crosslinking reaction. This binding mode is not only preferred for entropic reasons, but also might represent an optimal probe–enzyme alignment, which places both reactive groups in close proximity to the cysteine residues. In fact, variants with longer spacers have a reduced cytotoxicity in cell proliferation tests, which emphasizes less effective binding.[10] Superimposition of **4** onto the enzyme crystal structure shows a suitable distance of 10.4 Å for a modification by **4**, which likely induces a steric clash and prevents substrate catalysis (Figure S4 in the Supporting Information). Interestingly, the catalytically active cysteine residue (Cys303) was not alkylated by **4** or **6**.

To evaluate if this dual binding mode at a remote position can lead to inhibition of enzyme catalysis, we conducted ALDH1A1 activity assays with retinal or propanal according to published procedures.[18,21,23,25] These studies were first conducted with the recombinant enzyme to confirm its inhibition and were subsequently performed in A549 cell lysates to correlate the inhibitory effect with the biological phenotype. Pre-incubation of **4** and **6** in the reaction buffer allowed almost complete formation of the reactive duocarmycin cyclopropyl moiety (ca. 85 % for **6** to give the monocyclopropyl compound **7**, and 100 % for **4** to furnish the corresponding bis-cyclopropyl compound), which was subsequently added to the enzyme assay at various concentrations. Time-dependent inhibition of recombinant ALDH1A1 was detected for **6** ($IC_{50} = 41.0$ μM after 24 h pre-incubation), as well as for **4** ($IC_{50} = 26.3$ μM after 24 h pre-incubation, Figure S5 in the Supporting Information). As recombinant ALDH1A1 may not exhibit its full catalytic power and binding affinity, A549 cell lysates were directly incubated with **4** and **6** at various concentrations, and the residual enzyme

activity was monitored by the measurement of propanal turnover. Importantly, inactivation of ALDH1A1 in its native environment revealed a significantly stronger inhibition ($IC_{50} = 9.6$ μM after 14 h pre-incubation, $K_I = 22.6$ μM, and $k_{inact} = 0.114\ h^{-1}$) for **6** which remained constant over time. Compound **4** exhibited a much faster and stronger inactivation of the enzyme activity ($IC_{50} = 440$ nM after 75 min pre-incubation Figure 3c, which did not proceed any further, $K_I = 3.14$ μM, and $k_{inact} = 0.045\ min^{-1}$, Figure 3d). This pronounced difference is in line with the suggested binding mode and further supports the hypothesis that crosslinking of the two cysteine residues by **4** leads to a faster and more efficient inhibition of substrate turnover (Figure 3b, see also Figure S6 in the Supporting Information). Given the high abundance of ALDH1A1 in cells and the corresponding amount of protein that is required for the assay, which is around 5.3 pmol (Figure S7 and Table S4 in the Supporting Information), the IC_{50} value (440 nM = 22 pmol) emphasizes an only 4.2-fold excess of **4** over the enzyme. Moreover, as ABPP with **6** in intact cancer cells gave significantly stronger labeling relative to lysates (Figure 2a) as already mentioned, it is likely that ALDH1A1 partially loses activity after cell lysis. A direct ALDH1A1 inhibition assay with both compounds in living A549 cells failed, as immediate apoptosis and loss of activity within the duration of the assay occurred. Therefore, it is difficult to compare the in vitro enzyme inhibition data with acute cell toxicity (Figure S2 in the Supporting Information). Given the unique selectivity and high affinity of **4** and **6** for ALDH1A1, a direct link between cell viability and enzyme activity is in line with previous functional studies.[18,21–23]

In conclusion, we have shown that the duocarmycin family of anticancer compounds is potent, not only through an overstabilization of double-stranded DNA, but also by the inhibition of aldehyde dehydrogenase 1 as an essential enzyme target that plays important roles in the viability and detoxification of cancer cells. For the recently obtained and significantly more potent **4**, which does not bind DNA, ALDH1A1 inhibition is likely to be the preferred mechanism of action. However, ABPP target discovery does not allow the identification of molecules that are noncovalently bound to **4** and **6**. Therefore, it cannot be excluded that such an interaction contributes to the cytotoxicity of these compounds. However, this seems less probable based on the mode of action of **6** with double-stranded DNA, where a very fast, noncovalent interaction is followed by an alkylation. Therefore, these results contribute to our understanding of duocarmycins by providing insights into their protein-based mechanism of action.

Experimental Section

See the Supporting Information for details of the synthesis and characterization of compounds, bioassays, cell biology, as well as proteome preparation, labeling, and mass spectrometry.

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