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Duocarmycin Analogues without a DNA-Binding Indole Unit Associate with Aldehyde Dehydrogenase 1A1 and not DNA: A Reply

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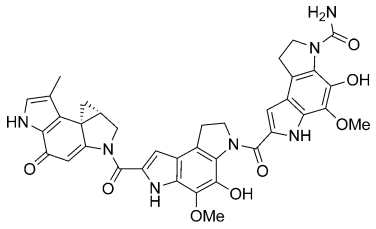
In 1978 scientists of the Upjohn company described a new antibiotic CC-1065 (**1**) with a remarkable cytotoxicty of about 30 pM, which they had isolated from the culture broth of *Streptomyces zelensis*;[1] later similar compounds as the

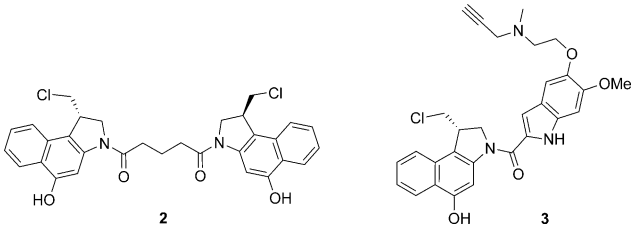


1: (+)-CC-1065

duocamycins and yatakemycin have been found. A characteristic feature of these compounds is their spirocyclopropylcyclohexadienone moiety that is connected to a substituted indole carboxylic acid by an amide bond.[2] The indole moiety causes an insertion of the compound into the minor groove of double-stranded DNA, and the spirocyclopropylcyclohexadienone moiety acts as an alkylating agent.

It has been shown that the alkylation is highly sequence-selective and takes place at N3 of an adenine moiety owing to a proximity effect.[2,3] Moreover, by using CD spectroscopy on living cells we elucidated, that at first in a very fast manner a noncovalent complex is formed reversibly, which is followed by a slower formation of a covalent C–N bond.[4] Recently, in our aim to develop novel prodrugs[5] for a selective treatment of cancer by using the antibody-directed enzyme prodrug therapy (ADEPT) approach we prepared analogues of seco-1,2,9,9a-tetrahydrocyclopropa[c]benzo[e]indol-4-one (seco-CBI) of type **2** where two of these molecules are connected by a diacid through two amide bonds. These compounds express an extraordinary high cytotoxicity of up to IC_{50} = 120 fM depending on the length of the diacid, though they do not contain the indole moiety usually believed to be necessary for a binding into the minor groove of DNA.[6] We have also prepared the enantiomer of one of these dimeric compounds showing a lower cytotoxicity by the factor of about 500.[7] For the investigation of the mode of action of the new dimeric seco drugs we analyzed their reaction with double-stranded oligonucleotides containing 15 bases in a 1:1 and 5:1 ratio by LC–MS spectrometry using a 7 Tesla-FTICR/MS system. By this method only covalently bound adducts would be detected. Therefore we also measured the induced Cotton effect using CD spectroscopy to identify noncovalent interactions.

In all experiments performed we did not see any kind of adduct of the dimeric seco-CBI compound **2** (Scheme 1) with the oligonucleotides. In a parallel experiment we also tested



2 **3**

Scheme 1. Structures of dimeric seco-CBI **2** and alkyne containing seco-CBI **3**.

compounds containing an indole moiety under the same conditions and they all showed the sequence-selective alkylation of adenine at N3 and a strong induced Cotton effect. We therefore concluded that for compounds of type **2** missing the indole moiety another target than double-stranded DNA must be responsible for their high cytotoxicity. For this purpose we prepared a seco-CBI derivative **3** containing an alkyne moiety (Scheme 1), which was used in activity-based protein profiling (ABPP) with the result that **3** besides binding to DNA also interacts with aldehyde dehydrogenase 1 (ALDH1A1).[8] For compound **3** and similar substances

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containing the DNA-binding indole unit we have always stressed that the interaction with the double-stranded DNA is the main reason for their cytotoxicity. However, owing to the lack of any interaction of the dimeric seco-CBI compound **2** with the tested oligonucleotides, we assumed that the binding to ALDH1A1 might be the cause of the observed cytotoxicity. However, in our paper we also pointed out that we cannot exclude noncovalent interaction with other biomolecules in the cell as source for their cytotoxity. Our view was confirmed by the recent synthesis of a novel dimeric seco-CBI drug containing an alkyne moiety and its use in ABPP experiments, where a strong binding to ALDH1A1 was observed.[11]

Tercel, Pruijn et al. have now repeated our work with additional experiments.[9] We have read their manuscript with great interest and thank the authors for their contribution, which initiates a scientific discussion about the targets of duocarmycin analogues. We very much appraise their effort, since our findings indeed contradict a long-existing dogma. To our great pleasure Tercel et al. could repeat our work in all aspects and the measured cytotoxicities match very well with our data even in the case of the dimeric seco-CBI compound with an $IC_{50} = 0.2$ pM. Moreover, they have also confirmed that the seco-CBI indole derivative **3** interacts with ALDH1A1. However, in their investigations they have found an alkylation of calf thymus DNA when using the dimeric seco-CBI **2**, and therefore claim that the target of the seco-CBI compound developed by us is DNA as in the case of **3**. Unfortunately, Tercel et al. used a highly artificial system with 0.1 μM of **2** and 250 μM of ctDNA in base pairs and even under these conditions the amount of alkylated product was rather low. Based on our calculations of the ring opening of CBI model systems with nucleophiles, we have seen that the reaction with N-nucleophiles as in DNA is faster than the reaction with OH^-.[6] Moreover, Tercel et al. did not check the sequence selectivity of the alkylating step. Furthermore, the results were not backed up by CD spectroscopy neither in vitro and nor in vivo. In addition, their own MS-based experiments lead to ambiguous results. While there were only traces of a dialkylated and thus crosslinked DNA detected by MS, a subsequent plasmid assay revealed migration shifts that are characteristic for DNA crosslinking. Since these in vitro DNA experiments are carried out under artificial conditions that may differ from the cellular system, the authors performed convincing whole-cell imaging to investigate native DNA binding. However, this was only done using a seco-CBI derivative containing a DNA-binding indole unit, which obviously showed a DNA binding. Owing to the lack of an alkyne moiety on the bifunctional CBI compound, without the DNA-binding indole unit, they could not disprove our hypothesis, thus leaving the question of cellular DNA binding of CBI compounds without indole unanswered.

We followed with interest the analysis of cytotoxicity across different cell lines. In fact, the amount of ALDH1A1 differs significantly across the panel tested. While our previous studies focused only on the analysis of A549 and the role of ALDH1A1 within these cells, it would be interesting to analyze the function of this enzyme in other cells as well. Reduced levels of protein do not automatically mean that this enzyme is less important for the cell. Again, we do not exclude that there might be other reversible (including nonproteinogenic) targets involved in CBI binding which contribute to biological effects. However, as long as the functional role of even little amounts of ALDH1A1 has not been proven in all cell lines, it is difficult to draw final conclusions.

Another interesting aspect of the work by Tercel et al. is the finding that the *R* and *R*,*R* enantiomers are less cytotoxic than the *S* and *S*,*S* enantiomers, respectively, which had also already been shown by us prior to the work of Tercel et al.;[6] however, the authors show a corresponding reduction of in vitro DNA crosslinking efficiency. Interestingly, also ALDH1A1 labeling by the *R* probe is significantly reduced to a background level. In contrast, the *R* probe gains affinity for several other, unidentified protein targets (as shown on the full gel in the Supporting Information). It is questionable if the residual weak protein band (obtained at a high probe concentration of 3 μM) belongs to ALDH1A1 or is a background protein as the authors suggest for the same band and similar intensity in their cell line comparison. The same is true for competitive experiments with *S*,*S* and *R*,*R* compounds, which are only meaningful if the identity of the weak band is assigned (if it is not ALDH1A1 it cannot be used for calculating ratios). In general we appreciate the labeling experiments by Tercel et al., since they verify that the chemical reactivity of the CBI alkylation unit alone is not sufficient for ALDH1A1 binding. The CBI unit requires the right stereochemistry for interaction with the enzyme, thereby emphasizing a specific binding mode. We feel that these results rather support than disprove the proposed selective interaction of the (*S*)-CBI unit to ALDH1A1.

To investigate enzyme inhibition the authors use an Aldefluor assay, which allows determining the intracellular aldehyde dehydrogenase activity by FACS analysis. We believe that this is an elegant strategy, since we observed a significant drop in enzyme alkylation after cell lysis, thus suggesting a low stability of the enzyme. According to the manufacturer protocol and a previous publication, this assay detects the activity of several aldehyde dehydrogenases and has no specificity for ALDH1A1.[10] Nevertheless, ALDH1A1 is highly abundant in A459, and the assay may thus give a good indication on intracellular ALDH1A1 inhibition. Owing to a lack of the recommended DEAB fluorescent background subtraction (manufacture protocol), the evaluation of irreversible inhibitors at a single time point, as well as the limited concentration range, it is not convincing to us that this assay appropriately reflects the real extent of intracellular ALDH1A1 inhibition by CBI analogues (no quantitative data are provided). However, the biggest concern about the performed inhibition assay is its insufficient comparability with the cytotoxicity assay. In both tests, cells are incubated with the compounds for 4 h, however, in the case of the cytotoxicity assays the cells are washed and cultured for 5 additional days. In contrast, immediate analysis is performed to evaluate ALDH1A1 inhibition. This is a crucial difference, since inhibition through irreversible inhibitors is a time-dependent process. Based on our studies with recombinant ALDH1A1 we know that full enzyme inhibition requires up to 24 h. Although the cells are washed after 4 h, inhibitors that

have entered the cells cannot be removed by this process. Based on the Supporting Information provided, 1000 A549 cells are seeded and exposed to compounds for cytotoxicity evaluation in a 96-well plate (about 100–200 μL). To the contrary, 500000 cells (in 1 mL) are investigated in the Aldefluor assay, which leads to a significantly increased amount of enzyme (50–100 fold) and a higher corresponding concentration for inhibition. Taken this into account, ALDH1A1 inhibition is likely to be present at compound concentrations lower than 30 nM. Thus, the results presented by Tercel et al. including the strict *S* stereo preference rather confirm ALDH1A1 as a target.

Finally, the title of the manuscript by Tercel et al. could be misleading, since it does not differentiate between duocarmycin analogues with and without the DNA-binding indole unit. Nobody is proposing that CC-1065 and similar compounds containing a DNA-binding unit do not interact with double-stranded DNA. We do not understand why Tercel et al. try to prove that there is no other target for all CC-1065 und duocarmycin analogues than DNA, although they have confirmed the interaction of **3** with ALDH1A1.

In conclusion, we agree with Tercel et al. that CBI analogues with a DNA-binding indole moiety bind to DNA and probably exhibit a large fraction of their bioactivity through this pathway as already published. It is also not at question that these molecules label and inhibit ALDH1A1 as confirmed by Tercel et al. as well. However, probes that lack the indole motif do not show cellular DNA binding, but still exhibit remarkable cytotoxicity.[11] Here, answers beyond the scope of DNA have to be provided. Open questions with regards to ALDH1A1 remain, and the analysis has to be extended to other biological systems as well. Thus far, additional reversible targets and additional pathways cannot be excluded.

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