Antitumor Agents

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Selective Treatment of Hypoxic Tumor Cells In Vivo: Phosphate Pre-Prodrugs of Nitro Analogues of the Duocarmycins**

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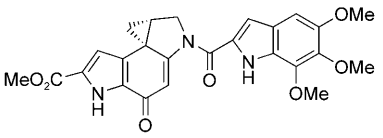
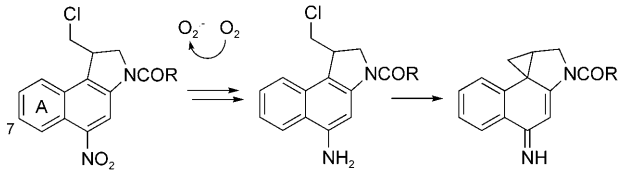
In memory of Francis Arthur Tercel and Naumai Aroha Mary Tercel

Duocarmycin SA (**1**) is one member of a small group of natural products that share a spirocyclopropylcyclohexadienone moiety and the following properties: binding in the minor groove of DNA, sequence-selective alkylation at N3 of adenine, and extreme cytotoxicity.[1] Several analogues have shown highly promising anticancer activity in animal models,[2] but all failed in clinical trial as humans proved to be very sensitive to the associated myelotoxicity.[3] Attempts to introduce a tumor-selective release or activation step have been widely pursued, including conjugation to tumor-specific antibodies[4] and the formation of glycosidic prodrugs.[5]

Our approach to introduce tumor selectivity is based on an amino analogue of the *seco* form of the alkylating subunit (**3** in Scheme 1), which has the potential to ring-close to an imino version (**4**) of the spirocyclopropylcyclohexadienone. AminoCBI (**3**) shares many of the same properties as the phenol congener, including sequence-selective DNA alkylation and potent cytotoxicity,[6] and can be formed by enzymatic reduction, in an oxygen-sensitive manner, of the prodrug nitroCBI (**2**). With appropriate A-ring substituents and side chains, **2** is up to several hundred times more toxic to hypoxic than to well-oxygenated cells.[7] Hypoxia is much more prevalent and severe in solid tumors than in normal tissue, and hypoxic tumor cells contribute to treatment failure as they tend to be chemoresistant, radioresistant, and highly malignant.[8] Hypoxia-activated prodrugs thus offer great promise for selective tumor therapy.[9] Application of **2** in vivo however requires more-water-soluble versions than those previously reported—the introduction of tertiary amino side chains resulted in only a marginal increase in solubility, and inactivity in vivo.[10] Herein we describe phosphate "pre-prodrugs" of **2** and their activity against hypoxic tumor cells.



Duocarmycin SA **1**



NitroCBI **2** AminoCBI **3** **4**

Scheme 1. Duocarmycin SA (**1**), and the proposed mechanism of action of hypoxia-selective nitroCBI prodrugs. R is a side chain that can bind in the minor groove of DNA.

Previous studies have shown that for high hypoxic selectivity, a primary or secondary sulfonamide or carboxamide at the 7-position of nitroCBI is favored.[7,10] A variety of alcohols **8**–**15** compatible with this design were prepared by EDCI-mediated coupling with the acids R^2CO_2H (Scheme 2). The indoline starting materials were either known (**5**) or prepared in a single step (**6**, **7**). For **7** an alternative and higher yielding route was developed based on hydrolysis of a primary carboxamide.[11] The nitroCBIs **10**, **11**, and **14** were reduced to the corresponding aminoCBIs **16**–**18** by hydrogenation or Zn reduction.

Phosphates were prepared via their *t*Bu esters which were cleaved in the final step of the synthesis to minimize handling of these highly polar compounds. The phosphate esters were prepared by reaction of an alcohol with di-*tert*-butyl-*N*,*N*-diisopropylphosphoramidite and subsequent oxidation, a strategy used to introduce the phosphate into either the side chain R^2[11] or the A-ring substituent (Scheme 3). Reaction of sulfonyl chloride **19** or acid **25** with amine **20** and cleavage of the trifluoroacetamide provided the indolines **21** and **26**, respectively, which were coupled with the acids R^2CO_2H as above, and the phosphate esters cleaved with trifluoroacetic acid (TFA).

Hypoxia-selective cytotoxicity in vitro was assessed with the cell-permeable alcohols rather than the highly polar

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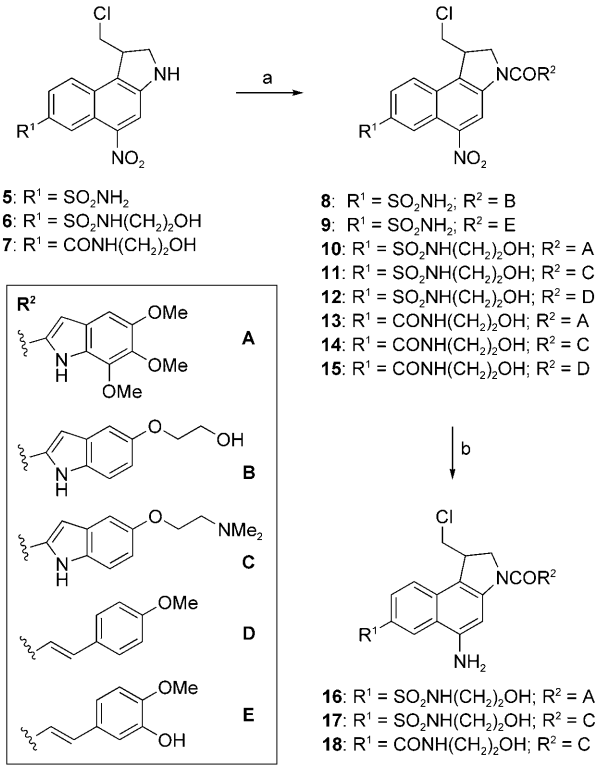


Scheme 2. Synthesis of nitroCBI and aminoCBI analogues bearing a free hydroxy group: a) R^2CO_2H, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI), toluenesulfonic acid (TsOH), dimethylacetamide (DMA), 51–88%; b) H_2, PtO_2, THF, 64–74% or (for **18**) Zn, NH_4Cl, acetone/water, 85%.

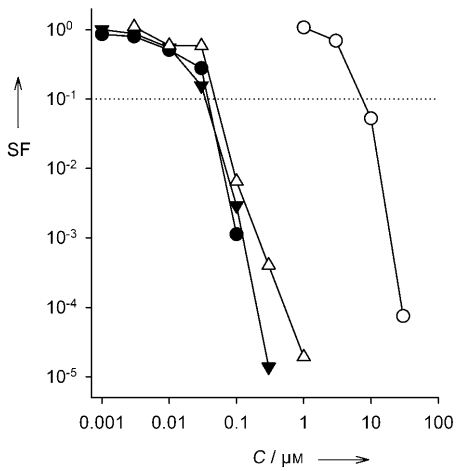
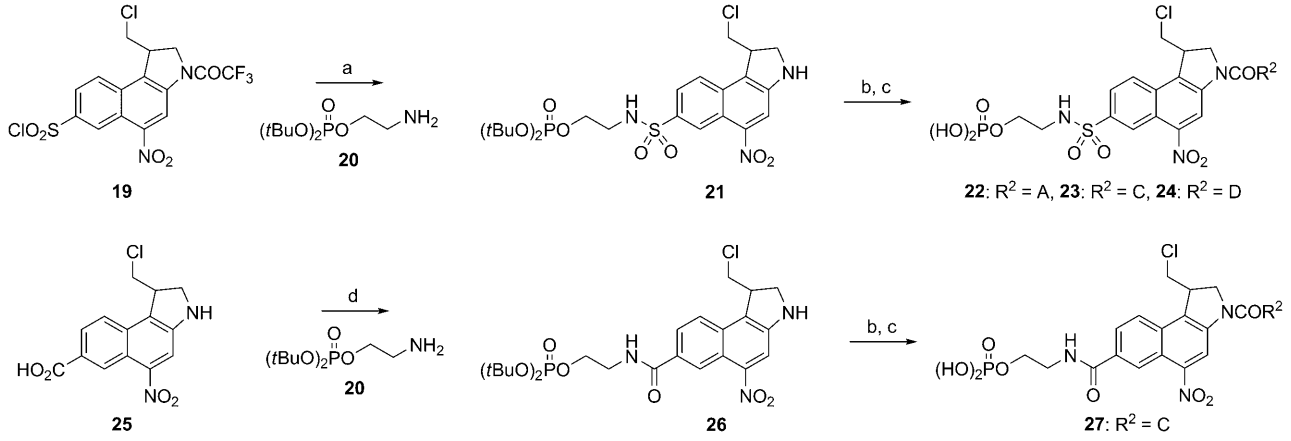


Figure 1. Cytotoxicity of nitroCBI **11** (open symbols) and aminoCBI **17** (filled symbols) in the human cervical carcinoma cell line SiHa, as determined by clonogenic cell killing. SF: surviving fraction; *C*: concentration; circles: oxic; triangles: hypoxic. Stirred cell suspensions were exposed to the compounds for 4 h under the specified gas phase and then plated. After 14 days incubation the number of colonies compared to untreated cells was used to determine SF. The interpolated concentration of **11** to cause 90% of the cells to be killed ($SF = 10^{-1}$, dotted line) was 7.5 μM under oxic conditions and 0.05 μM under hypoxic conditions.

phosphates. NitroCBI **11** for example, when exposed to suspensions of the human cervical carcinoma cell line SiHa, eliminated colony-forming cells about 150 times more effectively under hypoxic (< 20 ppm O_2) than oxic (20% O_2) conditions (Figure 1). Under hypoxia **11** becomes as toxic as the aminoCBI **17**, which displays no oxygen dependence in its toxicity. The alcohols **8**–**15** were compared by measuring IC_{50} values under oxic and hypoxic conditions and deriving a hypoxic cytotoxicity ratio [$HCR = IC_{50}(oxic)/IC_{50}(hypoxic)$]. Consistent with previous findings, HCR was sensitive to structural changes (possibly influencing ease of enzymatic reduction), and of the alcohols, **11** and **14**, bearing the basic side chain C, exhibited the highest selectivity.[11] NitroCBI **11** was further investigated in a panel of 14 human tumor cell lines of various tissue origin, and in every case significant HCRs were observed (Figure 2). Notably **17** again displayed no hypoxic selectivity, but potent cytotoxicity with an average IC_{50}(oxic) of 28 nM across the cell line panel.

Phosphates were prepared of nitroCBI alcohols that exhibited both low and high HCRs. In all cases the phosphates were much more water-soluble than the corresponding



Scheme 3. Synthesis of phosphate pre-prodrugs of nitroCBI: a) Et_3N, THF then Cs_2CO_3, MeOH, 91%; b) R^2CO_2H, EDCI, TsOH, DMA, 66–90%; c) TFA, CH_2Cl_2, 65–97%; d) benzotriazol-1-yl oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP), iPr_2NEt, 90%.

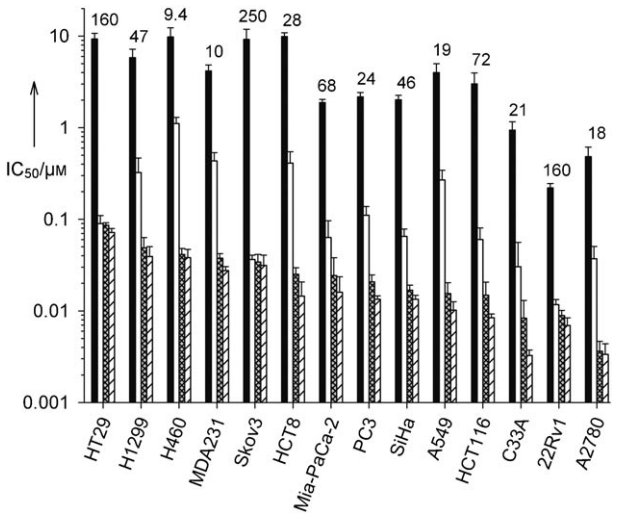


Figure 2. Cytotoxicity of nitroCBI **11** and aminoCBI **17** in a panel of human tumor cell lines, as determined by inhibition of proliferation. IC_{50}: concentration required to inhibit cell proliferation by 50%; black and white bars: **11** under oxic and hypoxic conditions, respectively; cross-hatched and hatched bars: **17** under oxic and hypoxic conditions, respectively. Cell monolayers were exposed to the compounds for 4 h under the specified gas phase and proliferation was measured 5 days later in comparison to untreated control cells. The numbers above the bars are the HCR values for **11** in each cell line.

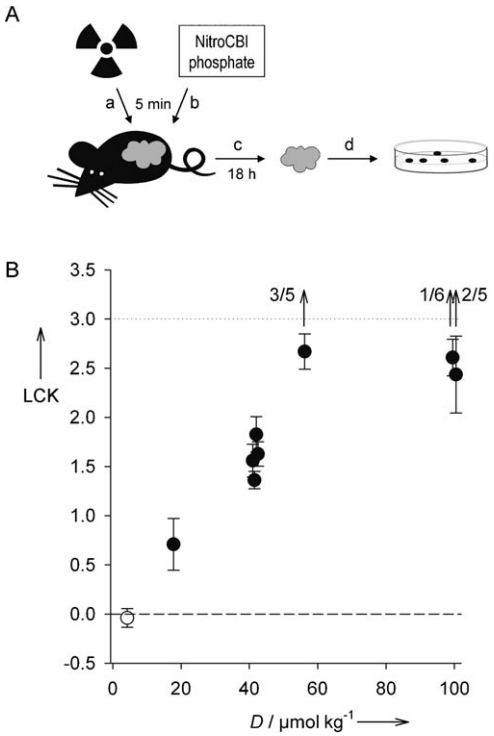


Figure 3. Antitumor activity in vivo by excision assay. A) Experimental design: Immunocompromised (CD1 nude) mice bearing SiHa xenografts were treated with a) a large single dose of γ irradiation (15 Gy), and 5 min later with b) an intravenous dose of a nitroCBI phosphate (or aminoCBI). After 18 h c) the tumor was excised, dissociated enzymatically, and plated d) to determine the number of surviving colony-forming cells per gram of tumor tissue. B) Antitumor activity of nitroCBI phosphate **23** (closed circles) and aminoCBI **17** (at its MTD; open circle). LCK (hypoxic log cell kill): *additional* cell kill for the combination treatment compared to radiation alone; *D*: dose; each point represents the mean ± SEM for groups of 5 or 6 animals. Dashed line: cell kill by radiotherapy alone; dotted line: approximate limit of quantitation; upward arrows: the number of tumors/groups for which no surviving clonogens could be detected.

alcohols. The solubility of **11** for example is only 4 μM in culture medium, but **23** can be dissolved in phosphate-buffered saline at > 4 mM, an increase in aqueous solubility of more than 1000-fold. Administration of **23** to mice and analysis of plasma samples showed that the phosphate is rapidly hydrolyzed to the alcohol, and that the plasma exposure to **11** at the dose used (42 μmol kg^{-1}) is more than enough to provide significant hypoxic cell kill, based on the in vitro assays.[11]

Administration of higher doses of **23** (> 100 μmol kg^{-1}) caused sporadic acute toxicity—some animals died within a few minutes, while others survived and exhibited no weight loss or any other signs of ill health over 60 days of observation. As a maximum tolerated dose (MTD) could not be defined the phosphates were instead compared at a nontoxic equimolar dose of 42 μmol kg^{-1}. Mice bearing a SiHa tumor were treated with a large dose of radiation, sufficient to cause about 99% of all cells to be killed (SF = 10^{-2}), as determined by excising the tumor 18 h after treatment and culturing the surviving cells. In other words, this radiation dose eliminates all but the 1 in 100 most hypoxic and radiation-resistant cells and any further killing of the resistant population when radiation is combined with a nitroCBI indicates activity against hypoxic tumor cells in vivo (Figure 3A). Of the phosphates the greatest (and highly significant) activity was seen with **23** and **27**, corresponding to the alcohols with the highest hypoxic selectivity in vitro.[11,12] Using the same assay phosphate **23** was also found to be highly active in five other human xenografts representing tumors of the cervix, colon, and lung.[11]

The activity of **23** was compared to that of aminoCBI **17** (Figure 3B). Unlike the prodrugs, **17** exhibited classical alkylating agent toxicity (reversible weight loss with a nadir at 7–10 days) and an MTD of 4.2 μmol kg^{-1}, but at this dose was completely inactive against hypoxic tumor cells. In contrast **23** caused dose-dependent hypoxic cell kill. At 56 μmol kg^{-1}, a nontoxic dose, the combination of **23** and radiation was so effective that in 3 of 5 animals the tumor was completely sterilized—within the sample size and detection limits of the assay (about 1 in 100000 surviving cells) no colony-forming cells could be detected. Phosphate **23** was further examined in a tumor growth delay assay (Figure 4). While **23** alone proved to be inactive, it significantly enhanced the effect of fractionated radiotherapy without apparent additional toxicity.[11]

In conclusion, the combination of a phosphate pre-prodrug strategy with our nitroCBI design converts non-selective and toxic DNA-alkylating agents into well-tolerated and water-soluble prodrugs that are highly selective for and active against hypoxic tumor cells in vivo. This approach offers great promise in tapping the anticancer potential of the

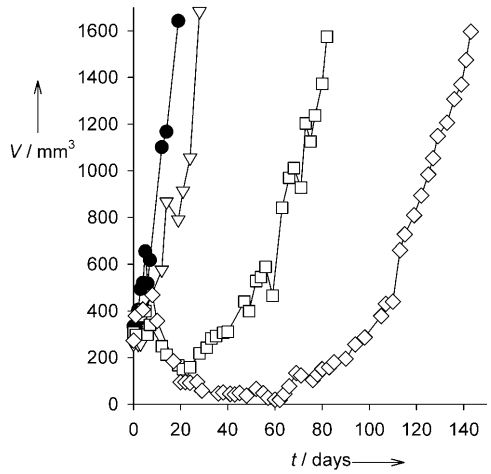


Figure 4. Antitumor activity in vivo by growth delay assay. Immunocompromised mice bearing SiHa xenografts were treated on days 1, 2, and 3 with **23** (42 μmol kg^{-1}) and/or γ irradiation (5 Gy). *V*: tumor volume of median mouse; *t*: time; circles: control; triangles: **23** alone; squares: radiation alone; diamonds: **23** 5 min after radiation. The time to quadrupling of the initial treatment volume was 16, 24, 77, and 128 days respectively.

duocarmycins and directing this action against the therapeutically most resistant tumor cell population.

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[11] See Supporting Information.
[12] Some activity was noted when phosphates were administered without radiotherapy. This may represent killing of the hypoxic fraction, or killing of oxic cells, either directly or by diffusion of aminoCBI from hypoxic zones.

