LEDSS, UMR CNRS 5616, Université Joseph Fourier, Grenoble, France[1], and Institute for Pharmaceutical Chemistry[2], Heinrich-Heine-University, Düsseldorf, Germany

A new 4-nitrobenzyl carbonate prodrug of methyl 5-benzyl-2-hydroxy-11-methylene-6-oxo-*5H*-benzo[*b*]carbazole-1-carboxylate for potential use with nitroreductase-based gene-directed enzyme prodrug therapy (GDEPT)

C. Asche[1], U. Kucklaender[2]

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Dr. Christian Asche, Université Joseph Fourier, LEDSS, BP 53, F-38041 Grenoble
Christian.asche@gmx.net

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A tumour-selective 4-nitrobenzyloxycarbonyl prodrug methyl 5-benzyl-2-hydroxy-11-methylene-6-oxo-5*H*-benzo[*b*]carbazole-1-carboxylate was synthezised for gene-directed enzyme prodrug therapy (GDEPT). The compound is a substrate for *E. coli* nitroreductase.

Gene-directed enzyme prodrug therapy (GDEPT) is a novel and promising approach to the tumour-selective treatment of cancer (McNeish et al. 1997; Denny 2002, 2003). One enzyme currently under investigation for GDEPT is the *E. coli* nitroreductase (NTR) which reduces in conjunction with its cofactor NAD(P)H the aromatic nitro groups of substrates to their corresponding hydroxylamines (Knox et al. 1992). In this context a 4-nitrobenzyloxycarbonyl spacer attached to the amino or hydroxyl groups of substrates has been developed which becomes self-immolative after enzymatic reduction (Carl et al. 1981; Mauger et al. 1994; de Groot et al. 2001).

We have previously reported on the synthesis, antitumour activity and structure-activity relationships of a series of novel 5*H*-benzo[*b*]carbazoles related to the ellipticines (Asche et al. 2005). Among them, particularly the unexpected very stable p-quinone methide **1** showed pronounced and promising *in vitro* antiproliferative effects in the National Cancer Institute's human tumour cell line screening panel comparable to those of well established anticancer agents (Monks et al. 1991). Thus, this compound seemed to be an attractive starting point for the development of a tumour-selective 4-nitrobenzyloxycarbonyl prodrug for the use with GDEPT. In our previous studies concerning the 5*H*-benzo[*b*]carbazoles it was shown that similar to the ellipticines an unprotected 2-hydroxyl group at the heterocycle increases antiproliferative activity, whereas acetylation or protection with a simple methyl group leads to much less active or even inactive compounds. Therefore, we assumed that protection of this hydroxyl group with a 4-nitrobenzyloxycarbonyl residue might also cause a significant reduction in toxicity.

The carbonate **2** was prepared without affecting the p-quinone methide moiety in 62% yield by acylation of the 2-hydroxyl group of **1** with 1 equivalent of 4-nitrobenzyl chloroformate at 0 °C in a pyridine-dichloromethane mixture and stirring the mixture for 10 h while warming to room temperature (Scheme) (experimental data available on request). To test the stability under physiological conditions, compound **2** was incubated in phosphate buffer (pH = 7.0) at 40 °C (data not shown). HPLC analysis of the incubation mixtures revealed that there were no significant changes in peak heights and peak areas of UV chromatograms over a period of 5 days indicating that this prodrug and especially its quinone methide and carbonate substructures are very stable. The same results were even obtained using methanol as solvent.

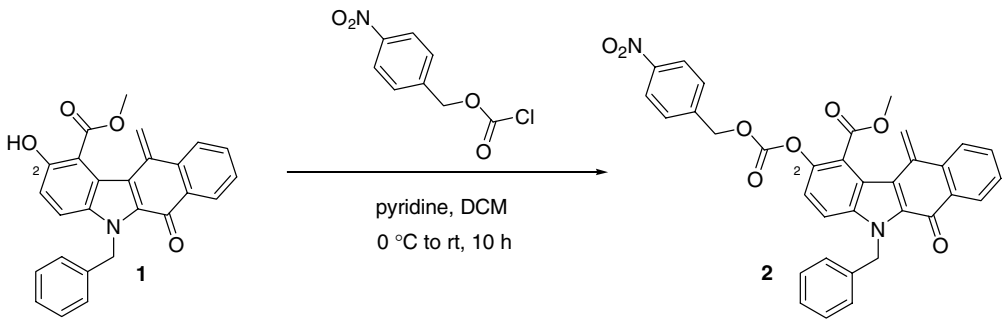
The *in vitro* anticancer properties of **1** have been confirmed in a second *in vitro* test by the NCI (Table). As expected from our previous studies and requested for a prodrug, compound **2** shows reduced antiproliferative activity at all levels (GI_{50}, TGI, LC_{50}) in comparison with

Table: Comparison of the *in vitro* anticancer activity of quinone methides 1 and 2[a]

Compound	MGM $-\log GI_{50}$[b]	MGM $-\log TGI$[c]	MGM $-\log LC_{50}$[d]
1[f]	6.42	5.88	5.25
1[g]	6.45	5.87	5.10
2	4.95	4.31	4.02

[a] Data obtained from the NCI's *in vitro* anticancer cell line screen (for details see ref. 5); [b] averaged log molar concentration for all tested cancer cell lines which led to 50% growth inhibition; [c] averaged log molar concentration for all tested cancer cell lines which led to total growth inhibition; [d] averaged log molar concentration for all tested cancer cell lines which led to 50% cell death; [e] the highest tested concentration (10^{-4} M) of the drug did not lead to 50% cell death; [f] first testing; [g] repetition of testing

Scheme



the corresponding parent drug **1**. At the LC_{50} level **1** exhibited an about 1 log unit stronger cytotoxic effect (MGM $-\log LC_{50}$ (**1**) = 5.25/5.10 vs. MGM$-$ log LC_{50} (**2**) = 4.02) and at the GI_{50} and TGI level it even led to an increase in growth inhibitory effects of about 1.5 log units (MGM$-$ log GI_{50} (**1**) = 6.42/6.45 vs. MGM$-$ log GI_{50} (**2**) = 4.95 and MGM$-$ log TGI (**1**) = 5.88/5.87 vs. MGM$-$ log TGI (**2**) = 4.31).

Furthermore, formation of the 2-hydroxyl compound **1** was observed upon incubation of prodrug **2** at a concentration of 10^{-5} M with 2 μg/ml enzyme and 5×10^{-5} M NADH in phosphate buffer at 37 °C which is a clear evidence that prodrug **2** is a substrate for for *E. coli* nitroreductase (data not shown).

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Faculty of Pharmaceutical Sciences, Kakatiya University, Warangal, India

Determination of valdecoxib in serum using a HPLC-diode array detector and its application in a pharmacokinetic study

S. Keshetty, R. K. Venisetty, V. Molmoori, V. Ciddi

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V. Ciddi University College of Pharmaceutical Sciences, Kakatiya University, Warangal – 506 009, AP, India
ciddiveeresham@yahoo.co.in

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A simple, sensitive, isocratic and reproducible reversed phase HPLC method for the determination of valdecoxib, a novel specific COX-2 inhibitor in human serum was developed using a diode array detector and celecoxib as internal standard. The system consisted of a C18 column and a detector set at 240 nm. The mobile phase was a mixture of acetonitrile : water acidified to pH 3.2 with orthophosphoric acid (OPA) (60 : 40) pumped at room temperature and a flow rate of 1 ml/min. The mean absolute recovery value was about 90%, while the intra ($n = 5$) and inter ($n = 5$) assay variations were $<18\%$. The calibration was linear over a concentration range of 20 ng/ml to 200 μg/ml with $r^2 > 0.999$. The limit of detection was ≤ 10 ng/ml. The method was used to study the pharmacokinetics of valdecoxib after a single dose oral administration to human volunteers.

Valdecoxib is a potent and specific COX-2 inhibitor approved by FDA for the treatment of rheumatoid arthritis, osteoarthritis and in primary dysmenorrhea. Valdecoxib has shown to be a highly selective and potent inhibitor of COX-2 in human whole blood and against the recombinant human enzyme. Valdecoxib has a mean absolute bioavailability of 83% (Bextra Valdecoxib Package Insert 2001) and is primarily metabolized by CYP 2C9 and CYP 3A4 enzymes (Yuan et al. 2002).

To date, two SPE-LC-MS-MS methods were developed and validated for the determination of valdecoxib and its metabolites in human urine (Zhang et al. 2003a) and plasma (Zhang et al. 2003b). An HPLC method using an UV-VIS detector was reported for the quantitation of valdecoxib in human plasma (Ramakrishna et al. 2004). Rao et al. (2005) reported the estimation of COX-2 inhibitors in pharmaceutical dosage forms and estimations in human plasma. Pavan Kumar et al. (2005) reported the estimation of valdecoxib and few other NSAIDS in human plasma. So far no simple HPLC method was reported for the determination of valdecoxib in human serum using UV-VIS or diode array detection. Here we report a simple, sensitive, reproducible and fast HPLC assay method for the determination of valdecoxib in human serum using UV detection. This method is applied to estimate the pharmacokinetics of valdecoxib after a single dose oral administration of valdecoxib tablet to human volunteers.