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Acknowledgement—We are indebted to Jenny Snoek-Liefrink, Piet J. van Assendelft and Jacqueline Dito for their support.

Eur J Cancer, Vol. 27, No. 3, pp. 250–253, 1991.
Printed in Great Britain

0277–5379/91 $3.00 + 0.00
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In vitro Antiproliferative and Metabolic Activity of Eight Novel 5-fluorinated Uracil Nucleosides

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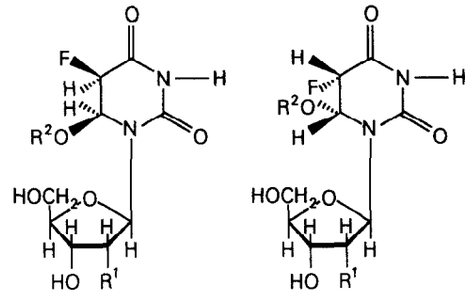
The *in vitro* growth inhibitory activity of eight novel 5-fluorinated uracil nucleosides was assessed in four human tumour cell lines, one of colon and three of head and neck squamous cell origin. These compounds are ribose or deoxyribose sugars with an acetoxy or an hydroxyl-group at the 6-position in the uracil part of the molecule, and their respective diastereoisomers. Antiproliferative effects were tested in an automated microculture assay based on the reduction of a tetrazolium dye, the MTT assay. Using a continuous drug exposure for four days, all novel nucleosides were more potent inhibitors of cell growth than 5-fluorouracil (5-FU). Most drugs were very active, having an IC_{50} value at least 10 fold lower than that of 5-FU, and this was consistently found for all cell lines. The 6-acetoxy compounds were generally more active than the compounds with a hydroxyl-group at the 6-position, while diastereoisomerism did not seem to influence the antiproliferative effect. Their capacity to inhibit the incorporation of tritiated deoxyuridine into DNA, which reflects the inhibition of thymidylate synthase, was measured in a short term assay. When tested at a concentration of 10^{-6} mol/l, most of the compounds were found to block this incorporation more efficiently than 5-FU.
Eur J Cancer, Vol. 27, No. 3, pp. 250–253, 1991.

INTRODUCTION

5-FLUOROURACIL (5-FU) has been used for several decades for the chemotherapeutic treatment of several types of cancer [1, 2]. In an attempt to improve the antitumour effect of 5-FU, several prodrugs have been synthesised. As such, we have evaluated doxifluridine (5′-deoxy-5-fluorouridine) in murine colon tumours and xenografts of head and neck squamous cell carcinomas and demonstrated that this drug has a better antitumour activity than 5-FU [3]. Other well known drugs of this class of agents are tegafur (N_1-tetrahydrofuran-2-yl-5-fluororacil) and floxuridine (5-fluoro-2′-deoxyuridine, 5-FUdR). Response rates with these drugs have been variable,

but especially when used in regional chemotherapy (e.g. intra-arterial infusions for liver metastases), doxifluridine [4] and 5-FUdR [5] show advantage over 5-FU.

Recently, the synthesis of three new classes of 5-fluorinated uracil nucleosides—6-acetoxy, 6-hydroxy and O^6, 5'anhydrocyclo adducts—was described [6, 7]. Upon heating in water it was found that the cyclonucleosides were slowly converted to their corresponding 5-fluoronucleoside, while the acetoxy derivatives were slowly converted to a mixture of the corresponding 5-fluoronucleoside and the 5-fluoro-6-hydroxy compound. These new compounds may potentially be cytotoxic in various ways; the phosphorylated forms of the ribonucleosides may be incorporated into RNA; the deoxyribonucleotide forms may either be incorporated into DNA or interfere with thymidylate synthase (TS). In addition, apart from the fact that the group at the 6-position creates a potentially interesting difference in lipophilicity with respect to that of the parent 5-fluoronucleosides, their chemical structure and behaviour are such that they may act as prodrugs of 5-FU either by enzymatic or by chemical degradation [6,7]. After the pilot *in vivo* study in athymic nude mice using ^{18}F as a tracer which showed that the cyclonucleosides were most probably converted instantaneously to their corresponding 5-fluoronucleosides [7], it was decided to give priority to the further development of the 6-acetoxy compounds and their corresponding solvolysis products, the 6-hydroxy compounds. The capacity of these nucleosides to inhibit the proliferation of *in vitro* growing monolayer cells was tested. In order to gain an initial insight in the mechanism of action of these new compounds, we measured the inhibition of the incorporation of radiolabelled deoxyuridine into DNA, reflecting the inhibition of TS, the target enzyme of 5-FU.



R^1	R^2	'South'	'North'
H	Ac	FOAc-UdR-s	FOAc-UdR-n
H	H	FOH-UdR-s	FOH-UdR-n
OH	Ac	FOAc-UR-s	FOAc-UR-n
OH	H	FOH-UR-s	FOH-UR-n

Fig. 1. Structural formulas of the 5-fluorinated uracil nucleosides. Diastereoisomers are designated as "South" (affix:-s, i.e. 5(R)-fluoro-6-(S)-OR² and "North" (affix:-n, i.e. 5(S)-fluoro-6-(R)-OR²). FOAc-UdR = 5-fluoro-6-acetoxy-deoxyuridine; FOAc-UR = 5-fluoro-6-acetoxy-uridine; FOH-UdR = 5-fluoro-6-hydroxy-deoxyuridine; FOH-UR = 5-fluoro-6-hydroxy-uridine.

MATERIALS AND METHODS

Cell lines

Three of the four human cell lines used were derived from squamous cell carcinomas from the head and neck (HNSCC). The line UM-SCC-14C is derived from a poorly differentiated HNSCC of the floor of mouth. UM-SCC-11B and HEp-2 are both derived from the larynx, being moderately and poorly differentiated, respectively. The UM-SCC cell lines were obtained from Dr T.E. Carey, University of Michigan, Ann Arbor, USA [8], whereas HEp-2 was purchased from Flow Laboratories (Herts, UK). The fourth cell line (WiDr) was established from a colon carcinoma and obtained from the American Type Culture Collection.

Cells were routinely grown at 37° C at 5% CO_2 as subconfluent monolayers in 25-cm^2 flasks (Nunclon, Nunc, Roskilde, Denmark). They were cultured in Dulbecco's modified Eagle's medium (DMEM, Flow), supplemented with 5% heat inactivated Fetal Calf Serum (FCS).

Drugs

Chemical names, abbreviations and structural formulas are described in Fig. 1. The 6-hydroxy and 6-acetoxy pyrimidine nucleosides were prepared and isolated as previously described [6,7]. 5-FU was obtained from Hoffmann–La Roche (Mijdrecht, The Netherlands); 5-fluoro-uridine (5-FUR) and 5-FUdR were from Sigma (St Louis, Missouri, USA). All drugs were dissolved in sterile aqua destillata and stored at −20° C as a 10^{-2} mol/l stock solution. Under these conditions all compounds were stable for several months.

In vitro growth inhibition

As a reproducible and sensitive index of cell growth we used a semi-automated microculture tetrazolium-based assay system, the MTT assay [9]; in this system the cell mediated reduction of a tetrazolium dye correlates very well with viable cell count [9–11].

Cells from routine cultures were transferred to 96-well flat-bottom plates (Greiner, Nürtinger, Germany). Optimal test conditions for each cell line had to be determined. To ensure the growth of untreated cells was exponential during the whole period of drug incubation (4 days), we plated 4000 cells/well for UM-SCC-11B and 2000 cells/well for the other three cell lines. In order to permit chemosensitivity testing of exponentially growing cells, drugs had to be added after a lag phase which varied from 24 h for HEp-2 and UM-SCC-14C to 48 h for UM-SCC-11B and WiDr. During the lag phase the cells were cultured in 150 µl standard medium (DMEM + 5% FCS) and thereafter 50 µl drug containing medium (without drug for the control) was added. The final drug concentration in the wells varied from 10^{-4} to 10^{-9} mol/l. After 4 days of exposure 30 µl MTT ([3-4,5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazoliumbromide], Sigma) was added to each well (final concentration 0.5 mg/ml); the stock solution of MTT was made in phosphate buffered saline and stored at −20° C in the dark [11]. After mixing the plate for 30 s on a horizontal plate shaker, the cells were recultured for 2 h; the medium was removed and dimethylsulphoxide (Merck, Darmstadt, Germany) containing 0.5% FCS [9] was added (150 µl/well) to solubilise the reaction product, formazan. After thoroughly vibrating on a plate shaker (5 min), the absorbance of each well was measured using a microplate reader (Titertek, Multiskan MCC/340 from Flow) at 540 nm. Background (medium with drugs) subtraction was

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Revised 23 Nov. 1990; accepted 3 Dec. 1990.

performed automatically. The experimental set up was such that in a single experiment one tumour cell line was tested for its sensitivity to all drugs at all concentrations. All values were averages from 4 different wells. From each experiment the IC_{50} was estimated, i.e. the concentration at which the optical density is 50% of that of the untreated control. For this calculation the optical density values at the start of the drug incubation were subtracted from the values obtained after the four days period of drug exposure. Each experiment was repeated at least three times.

Incorporation of [^{3}H]UdR into DNA

Measurement of [6-^{3}H]-deoxyuridine ([^{3}H]UdR) incorporation into DNA of cultured WiDr cells was basically performed as described by Peters *et al.* [12]. WiDr cells were chosen because they are extensively characterised concerning 5-FU metabolism [12–14]. Cells (1.5×10^5/well) were incubated in DMEM containing wells of Millititer GV plates (Millipore Corporation, Bedford, Massachusetts). After a preincubation of 30 min cells were exposed to the drugs (added in 15 μl medium) for 1 h at a concentration of 10^{-6} mol/l. Next, 10 μl of [6-^{3}H]UdR [0.1 nmol/(37 kB*q*), Amersham, UK] was added. After 2 h the medium was filtered through the membrane of the wells using a vacuum pump. Subsequently, the macromolecules were precipitated on the filters by the addition of 150 μl ice-cold 5% trichloroacetic acid. Acid soluble material was filtered through the membrane and the filters were washed four times with water and subsequently with 70% ethanol. After drying, the filters were collected with a Millititer filter punch; the precipitated material was solubilised overnight with 2 mol/l NaOH and radioactivity was counted in Dimilume (United Technologies Packard, Groningen, the Netherlands). Corrections were performed for non-specific binding of [^{3}H]UdR.

RESULTS

The growth inhibiting activity of eight recently developed 5-fluorinated uracil nucleosides was analysed in four human tumour cell lines by means of the MTT assay. This tetrazolium dye based assay gave reproducible results with these cell lines; during the four day period of drug exposure, the optical density measured in the wells with untreated cells increased to values ranging from 0.6 to 1.2, thus permitting the reliable calculation of IC_{50} values.

In the four cell lines all drugs tested were more potent in their antiproliferative effect than 5-FU (Table 1, Fig. 2). Interestingly, the cell lines differed strongly with respect to their sensitivity to 5-FU: HEp-2 had a 50 fold higher IC_{50} than another HNSCC cell line UM-SCC-14C. In order to survey the potency of the new derivatives as compared to 5-FU, Fig. 2 shows the data relative to that of 5-FU. 5-FUR and 5-FUdR were the most active compounds in our test system; in two cell lines (WiDr and UM-SCC-14C) the IC_{50} values for 5-FUdR was even lower than 1 nmol/l. As for the novel derivatives, the acetoxy diastereoisomers were generally more active than the corresponding hydroxy isomers; in 11 out of 14 comparisons (ranked according to class of nucleoside) the acetoxy derivatives showed a lower IC_{50} value ($P<0.05$, Student's *t* test). Comparison of the activities of deoxyribose- vs. ribose-nucleosides did not provide consistent results. In 5 out of 14 cases the deoxy compounds were significantly more active, but for the WiDr line both acetoxyribonucleosides were more antiproliferative than the corresponding deoxyribose derivatives. Within the experimental error, diastereoisomerism did not appear to have a pronounced influence on the antiproliferative effect.

Table 1. Comparison of growth inhibiting effects of 5-fluoro-pyrimidines in four cell lines

Drugs	UM-SCC-14C	HEp-2	UM-SCC-11B	WiDr
5-FU	615 (64)	30750 (6899)	5800 (923)	3730 (265)
5-FUR	16 (2)	110 (45)	40 (23)	25 (3)
FOAc-UR-n	140 (46)	270 (135)	400 (47)	15 (2)
FOAc-UR-s	60 (23)	250 (25)	337 (72)	18 (1)
FOH-UR-n	500 (152)	6000 (865)	3170 (970)	1370 (294)
FOH-UR-s	475 (20)	6000 (865)	3300 (890)	1500 (352)
5-FUdR	<1	53 (4)	11 (1)	<1
FOAc-UdR-n	28 (1)	366 (33)	70 (18)	190 (20)
FOAc-UdR-s	28 (11)	466 (66)	95 (30)	440 (114)
FOH-UdR-n	250 (76)	n.t.	1060 (663)	n.t.
FOH-UdR-s	88 (30)	1833 (439)	242 (106)	240 (23)

IC_{50} values (nmol/l) are as determined with the MTT-assay (four days drug exposure). Average (S.E.) values are based on at least three separate experiments.
n.t. = not tested.

As a first step in revealing the mechanism of action of these new derivatives, we investigated the potential capacity of these drugs to form a nucleotide being able to inhibit TS. This was approached in an indirect way by measuring the inhibition of the incorporation of [^{3}H]UdR into DNA of WiDr cells. Because of the short incubation time, a relatively high concentration of drugs compared to the IC_{50} was used. Apart from FOH-UR-n and FOH-UR-s (Fig. 1), all compounds were able to block [^{3}H]UdR incorporation much more efficiently than 5-FU (Fig. 3). In general, the deoxyuridine derivatives were found to be more potent than the uridine compounds, while the formation of a complex with TS did not seem to be influenced by diastereoisomerism, i.e. the difference in position of the F atom at C_5 and that of the group at the C_6.

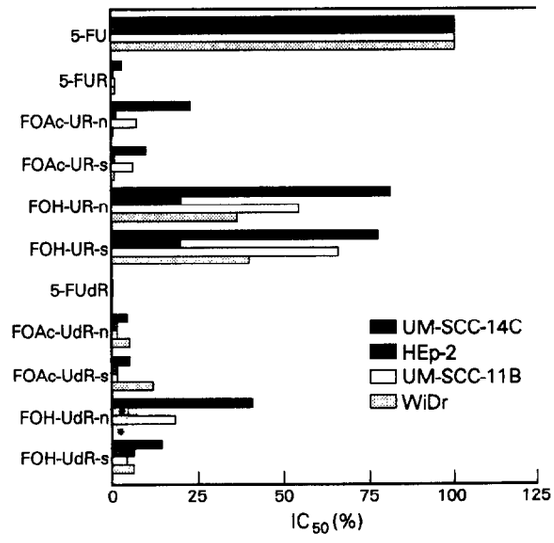

Fig. 2. IC_{50} values of 5-fluorinated nucleosides as determined with the MTT assay (four days drug exposure). Values are shown as percentage of the IC_{50} of 5-FU. * = not tested.

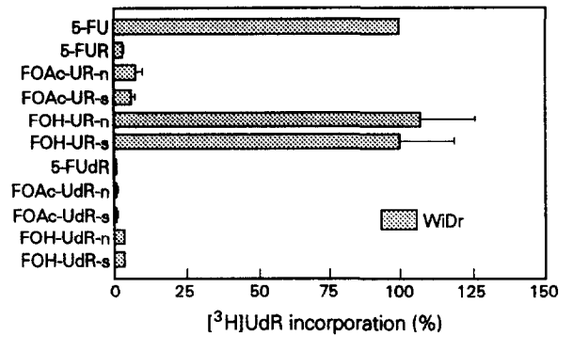

Fig. 3. [^{3}H]UdR incorporation into DNA of WiDr cells after 3 h exposure to various nucleosides at a concentration of 10^{-6} mol/l; the last 2 h of the incubation, [^{3}H]UdR was present. Inhibition of [^{3}H]UdR incorporation in the presence of 5-FU was 50% (down to 127 000 dpm per 1.5 × 10^6 cells). Values represent the mean (S.E.) and are expressed as a percentage of the [^{3}H]UdR incorporation by 5-FU.

DISCUSSION

The novel fluoronucleosides not only are more active than 5-FU, but they also show a distinct difference in activity when compared to their parent 5-fluoronucleosides. This indicates that the novel compounds—at least *in vitro*—are not instantaneously converted to 5-FU or their parent 5-fluoronucleosides. In addition, the compounds bearing an acetoxy group at the 6 position were generally more effective than those bearing an hydroxy group. So, considering this difference in cytotoxicity between these groups of compounds, it seems unlikely that the FOAc compounds are first degraded to the FOH compounds to be active [6, 7].

Fluoropyrimidines have a complicated mechanism of action, which varies in different cells and tumours [2, 12–14]. One of the metabolic pathways responsible for cellular death is the formation of a ternary complex as FdUMP with the folate cofactor and TS, thus inhibiting DNA synthesis. Our results in the WiDr cell line, which has been characterised with respect to the metabolism of 5-FU [12–14], show that the [^{3}H]UdR incorporation into DNA is inhibited effectively by most of the compounds (Fig. 3). The deoxy derivatives inhibit this [^{3}H]UdR incorporation more efficiently than the uridine compounds, while the two compounds that were the least cytotoxic, i.e. FOH-UR-n and FOH-UR-s, were also poor inhibitors of [^{3}H]UdR incorporation. This indicates that the cytotoxicity of the novel compounds is mediated—at least partly—by the inhibition of TS. Moreover, the observation that the deoxy derivatives block TS more effectively than 5-FU seems to support the hypothesis that these compounds are directly converted to their mononucleotide derivatives, which inhibit TS; conversion to 5-FU prior to e.g. FdUMP is unlikely considering the less inhibitory activity of 5-FU on the [^{3}H]UdR incorporation in WiDr cells. However, it is clear that more studies have to be performed to confirm the relevance of TS-inhibition in the cytotoxic effect when cells are exposed to the drugs at various concentrations and longer than the 3 h exposure used in the assay in this study. For the WiDr cells, for example, it seems very likely that the FOAc-UR derivatives also act via incorporation into RNA: although being a less potent inhibitor of TS than FOAc-UdR, the FOAc-UR derivatives have a 10-fold lower IC_{50} value than the FOAc-UdR compounds.

In conclusion, a series of novel fluorinated uracil nucleosides is described that have a better antiproliferative activity *in vitro* than 5-FU. Most of these compounds are able to block the incorporation of [$_3$H]UdR into DNA in an efficient way.

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Acknowledgements—This study was supported by grants from the "Maurits en Anna de Kock stichting" and the "Nijbakker-Morra stichting". The authors would like to thank Drs G. van Dongen, J. Herscheid and A. Kegel for their contribution. Dr G.J. Peters is a senior fellow of the Royal Netherlands Academy of Sciences.