

CONCENTRATION AND TIME DEPENDENT GROWTH INHIBITION AND METABOLISM IN VITRO BY 2',2'-DIFLUORO-DEOXYCYTIDINE (GEMCITABINE)

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INTRODUCTION

2',2'-Difluoro-deoxycytidine (dFdC) is a new deoxycytidine analogue. 1- β -D-arabinofuranosylcytosine (cytosine arabinoside, ara-C), also a deoxycytidine analogue, is one of the most effective agents against acute myelogenous leukemia (AML). However, activity against solid tumours both in preclinical systems and patients is limited (1). As opposed to this, dFdC has shown excellent antitumour activity in various preclinical solid tumour model systems (2,3). Several phase I trials using different schedules have been completed (4,5) and the drug is now being evaluated in some clinical phase II trials. Some activity has been observed in patients with non-small-cell lung cancer, ovarian cancer and pancreas cancer. Ara-C exhibits its antitumour effect through accumulation of its triphosphate ara-CTP, which is subsequently incorporated into DNA, leading to growth inhibition (1,6). The antitumour effect of ara-C is dose and time dependent (7). The phosphorylation of ara-C to ara-CMP, catalysed by deoxycytidine kinase (dCK), is the crucial step. The drug can be inactivated by deamination to ara-U, catalysed by deoxycytidine deaminase (dCDA) (1). Like ara-C, dFdC must be converted into dF-dCMP, catalyzed by dCK, and subsequently to dF-dCTP. dFdC can be inactivated by deamination to dFdU (difluorodeoxyuridine), catalysed by dCDA (8,9). Until now, most studies on dFdC have been limited to leukemic cell lines. Because of the promising preclinical results in head & neck, colon and ovarian cancer, we used these solid tumour cell lines as a model for solid tumours. We determined in vitro whether sensitivity to the drug and accumulation of dF-dCTP were time and concentration dependent.

MATERIALS AND METHODS

Cell culture. The following cell lines were used: C26-10 and WiDr, a murine and a human colon carcinoma, respectively; A2780 and OVCAR-3, both human ovarian carcinomas; UM-SCC-22B and UM-SCC-14C (22B and 14C), both human head and neck squamous cell carcinomas. The cells were cultured in Dulbecco's Modified Eagle's Medium (Gibco), supplemented with 10% v/v heat inactivated Foetal Calf Serum (FCS) (Gibco), 1 mM L-glutamine (Sigma) and 250 ng/ml gentamycin, at 37°C,

at 5% CO₂. For growth inhibition studies the culture medium was supplemented with 5% v/v FCS.

Chemosensitivity testing. For assessment of chemosensitivity, on day 0 cells were seeded in triplo in flat bottom 96-well plates. After 24 h dFdC was added in a concentration range from 10⁻⁴ to 10⁻¹¹ M. After exposure for 0.5, 1, 2, 4, 24, 48, and 72 h, the cells were washed twice and allowed to grow till day 4. At day 1 and 4 the sulforhodamine B (SRB) protein assay was performed as described earlier (10,11), to quantify the number of cells. Growth inhibition was expressed as IC₅₀: the concentration of dFdC, which caused 50% growth inhibition.

dF-dCTP accumulation. dF-dCTP accumulation was measured in exponentially growing cells, after exposure for 1, 4, or 24 h to 1, 10 and 100 μM dFdC. Cells were harvested and counted. Nucleotides were extracted using trichloro-acetic acid as described earlier (12). The HPLC analysis for the nucleotides was performed with a Partisphere SAX anion exchange column, using isocratic elution (13). The HPLC analysis for dF-dCTP was performed essentially as described by Heinemann et al. (9).

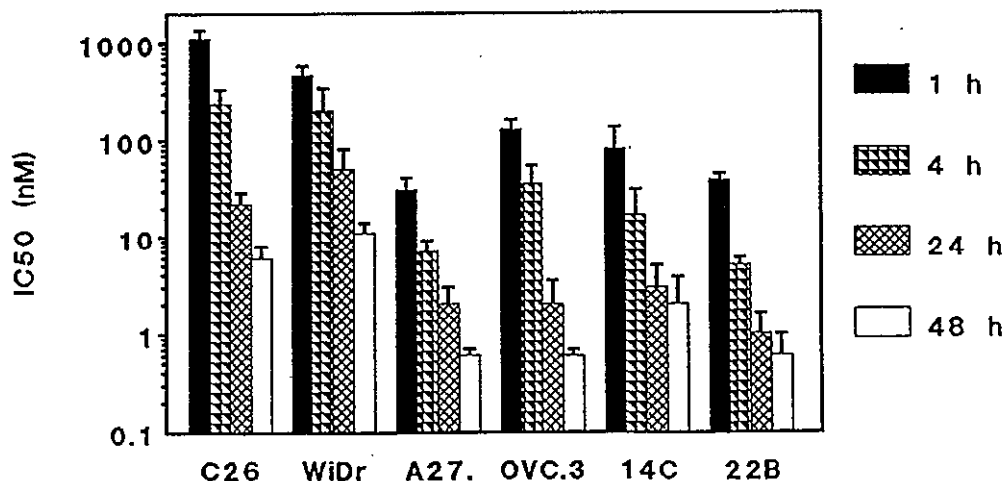


Fig. 1 IC₅₀'s of dFdC at different exposure times, in 6 tumour cell lines. Values are means ± SD of 4-10 separate experiments.

RESULTS

Growth inhibition. In all cell lines the growth inhibitory effect of dFdC was time and concentration dependent (Fig. 1). The effect of 30 min exposure was not significantly different from that of 1 h exposure. Similar results were obtained after 2 and 4 h and 48 and 72 h exposure, respectively. Therefore only data of the 1, 4, 24 and 48 h exposure are presented. Although in all cell lines a comparable time dependence was observed, the absolute IC₅₀'s varied considerably between the different cell lines (Fig. 1). For example for the 24 h exposure, the IC₅₀ in the colon tumour cell line WiDr was 3x higher than in the squamous cell carcinoma 14C, and 26x higher than in the ovarian carcinoma A2780.

dF-dCTP accumulation. The accumulation of dF-dCTP, as determined in C26-10 and WiDr, was concentration dependent (Fig. 2). For example in C26-10, 24 h exposure to 100 μ M dFdC yielded a 16x higher dF-dCTP concentration than 24 h exposure to 1 μ M. More pronounced, however, is the time dependence of dF-dCTP accumulation. 24 h Exposure of C26-10 cells to 100 μ M of dFdC yielded 43x more dF-dCTP than 1 h exposure. The normal nucleotide pattern changed by dFdC exposure. The major disturbances were observed in the UTP and CTP pools of WiDr. In this cell line CTP decreased to undetectable levels after 24 h exposure to dFdC, and that of UTP increased considerably. In C26-10 cells UTP accumulation was also seen, but the CTP pool did not change significantly.

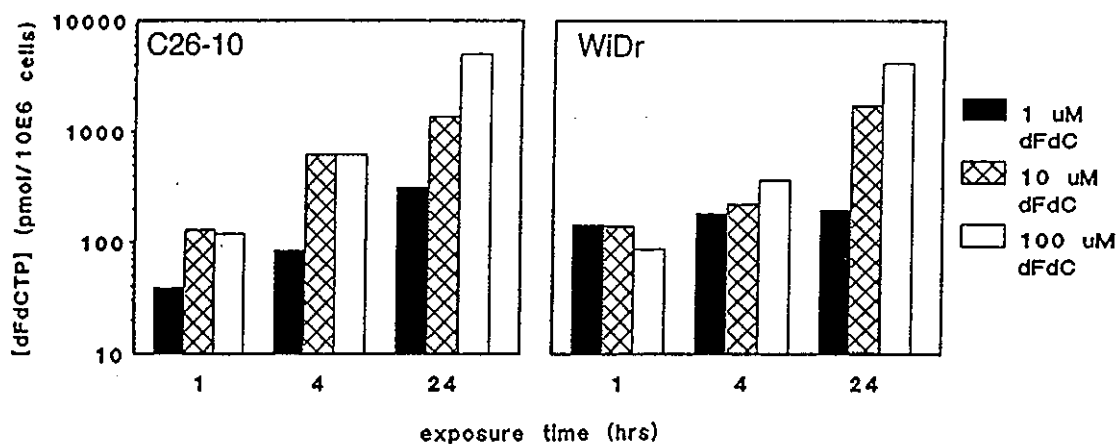


Fig. 2 dF-dCTP accumulation in C26-10 and WiDr cells, after continuous exposure to 1, 10 and 100 μ M of dFdC for 1, 4 and 24 h. Data are from one experiment, performed in triplo.

DISCUSSION

The growth inhibitory effect of dFdC in solid tumour cell lines is concentration and time dependent. This also holds for the accumulation of the active triphosphate dF-dCTP. It appears that the antitumour effect of dFdC is related to the dF-dCTP accumulation, as it is for ara-C and ara-CTP. It is known that dF-dCDP can inhibit ribonucleotide reductase, resulting in a depletion of dCTP (14,15). As dCTP is the most important feedback inhibitor of dCK, a depletion of dCTP would result in a higher dCK activity. The concentration dependence of dF-dCTP accumulation becomes more pronounced with longer exposure (Fig. 2). Possibly at 1 and 4 h exposure dCK is still feedback regulated by dCTP. After 24 h, however, enough dF-dCDP could be formed to deplete dCTP, resulting in a higher dCK activity. Under these circumstances dFdC may be phosphorylated more efficiently at low concentrations. The observed increase in UTP at longer exposure may also enhance dCK activity, since UTP is a better substrate than ATP (16). Interestingly, the difference in dF-dCTP concentration after 24 h exposure between 10 μ M and 100 μ M dFdC is much smaller than the difference between 1 and 10 μ M, after 24 h exposure. This could be due to saturation of the phosphorylating enzymes. Gr \ddot{u} newald et al. (5) recently published similar findings in patients treated with dFdC. Longer infusion times with the same absolute dose resulted in higher concentrations of dF-dCTP in the mononuclear cells. But there was a limit to the dose dependency; accumulation of dF-dCTP did not increase at doses >900 mg/m², probably due to saturation of the phosphorylating enzymes (5,17). From our *in vitro* results and literature data it seems therefore that dFdC would exhibit a better antitumour effect when applied by continuous infusion. It is not clear how

dFdC causes CTP depletion in WiDr cells, when exposed for 24 h. From this CTP depletion in WiDr and the UTP accumulation in both WiDr and C26-10 it seems that dFdC or one of its metabolites may inhibit CTP synthetase, an enzyme which catalyzes the synthesis of CTP from UTP. We conclude that the growth inhibitory effect of dFdC is concentration and time dependent and is related to the accumulation of its triphosphate derivative dF-dCTP.

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