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2′,2′-Difluoro-deoxycytidine (gemcitabine) incorporation into RNA and DNA of tumour cell lines

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Abstract—Gemcitabine (dFdC) is a new cytidine analogue which is active mainly by the incorporation of its triphosphate (dFdCTP) into DNA, leading to cell death. We determined incorporation of dFdC into nucleic acids of two solid tumour cell lines: the murine colon carcinoma cell line Colon 26-10, the human ovarian carcinoma cell line A2780, and the human leukemic cell line CCRF-CEM. dFdC was not only incorporated into DNA, but also into RNA. The extent of incorporation into DNA was highest in A2780 cells and lowest in CCRF-CEM cells (2–4-fold difference). The same pattern was observed for incorporation into RNA, but with a 10–20-fold difference. In A2780, incorporation into DNA was about twice that of the incorporation into RNA, in CEM cells 10–20-fold that of RNA. Incorporation into RNA was verified using two methods for separation of RNA and DNA, acid precipitation and CsCl-gradient centrifugation. Incorporation into DNA was time and concentration dependent, but incorporation into RNA seemed to be only concentration dependent. We also determined the effect of dFdC on DNA and RNA synthesis by measurement of thymidine and uridine incorporation, respectively, using similar conditions as for the incorporation studies. In all three cell lines DNA synthesis was inhibited almost completely, even at 0.1 μM dFdC and at 4-hr exposure. RNA synthesis inhibition did not exceed 50% in both solid tumour cell lines, even at 1 μM dFdC exposure for 24 hr. A clear concentration effect was only observed in the CCRF-CEM cell line and only after 24 hr exposure. At a 1 μM dFdC exposure for 24 hr, RNA synthesis was completely inhibited in these cells. Incorporation of dFdC into RNA and inhibition of RNA synthesis represent an unrecognized but possibly important mechanism of action of this drug.

2′,2′-Difluorodeoxycytidine (dFdC,* gemcitabine) is a new cytidine analogue, which, unlike 1-β-D-arabinofuranosylcytosine (ara-C), has shown excellent anti-tumour activity *in vivo* against a variety of murine solid tumours and human tumour xenografts [1, 2]. Promising clinical activity has been observed in Phase I and II trials with adenocarcinomas of the lung and ovarian cancer [3–6]. The mechanisms of action of dFdC are not completely understood. Recent studies have shown that, like ara-C, dFdC must be phosphorylated to the active triphosphate dFdCTP, which can be incorporated into DNA leading to cell death [7–9]. These studies were performed in CCRF-CEM cells, a human T-lymphoblastoid cell line [9] and K562, a human chronic myelogenous leukemia cell line [10]. No incorporation of dFdC into RNA nor inhibition of RNA synthesis was reported in these studies. Preliminary studies on dFdCTP accumulation in cell lines derived from colon cancer and ovarian cancer demonstrated a concentration and time dependence of dFdCTP accumulation [11], which correlated with the sensitivity to dFdC. Here we report that dFdC can not only be incorporated into DNA, but also into RNA. Furthermore dFdC inhibits both DNA and RNA synthesis.

Materials and Methods

Materials. dFdC and [5-^{3}H]dFdC (16.7 Ci/mmol) were a kind gift of Eli Lilly Inc. (Indianapolis, IN, U.S.A.) [2-^{14}C]Thymidine ([^{14}C]TdR, 58.8 Ci/mol) was purchased from Dupont de Nemours NEND (Dreiech, Germany) and [5-^{3}H]uridine ([^{3}H]UR, 27.8 Ci/mmol) from Amersham International (Amersham, U.K.). Guanidine isothiocyanate (GIT) (enzyme grade) was purchased from Ultra Pure BRL, Life Technologies Inc. (Gaithersburg, MD, U.S.A.). Cesium chloride (reagent grade) was purchased from Gibco BRL (Paisley, U.K.) RNAse A (DNase free and boiled prior to use) was purchased from Boehringer Mannheim (Mannheim, Germany). All other chemicals were p.a. grade and commercially available.

Cell culture. Human ovarian carcinoma A2780 [12] and murine colon carcinoma C26-10 cells [13] were maintained in exponential growth in Dulbecco's modified Eagle's medium (Gibco) supplemented with 5% heat-inactivated foetal calf serum (Gibco), 1 mM L-glutamine (Sigma Chemical Co. St Louis, MO, U.S.A.) and 250 ng/mL gentamicine at 37° and 5% CO_2. Human T-lymphoblastic CCRF-CEM cells (American Type Culture Collection, Rockville, MD, U.S.A.) were maintained in suspension culture in exponential growth in RPMI 1640 medium (Gibco) supplemented with 10% heat-inactivated foetal calf serum and 250 ng/mL gentamicine at 37° and 5% CO_2.

Determination of dFdC incorporation into DNA and RNA. Incorporation of dFdC into DNA and RNA was measured using two different approaches. One assay was based on acid precipitation of nucleic acids, followed by enzymic separation of RNA and DNA. This assay was performed essentially as described previously [14]. Briefly, cells were cultured in 6-well plates, and exposed to 0.1 or 1 μM [5-^{3}H]dFdC (4 Ci/mmol) for 4 and 24 hr. After exposure, A2780 and C26-10 cells were trypsinized, CEM cells washed once. Then cells were counted, washed and resuspended in 100 μL phosphate-buffered saline (PBS), followed by addition of 100 μL of 0.8 M $HClO_4$, mixed and chilled on ice for 20 min. After centrifugation for 5 min at 10,000 *g* the pellet was washed three to four times with PBS, resuspended in 100 μL PBS, and incubated for at least 15 min at room temperature with 2 μL of 2 mg/mL RNAse A (final concentration 2 μg/10^6 cells). Then 30 μL of 5 M $HClO_4$ were added and the mixture was chilled on ice for 5 min. After centrifugation at 10,000 *g* for 3 min, the supernatant containing the hydrolysed RNA nucleotides was counted for 5 min in a Kontron liquid scintillation counter in 10 mL Dimilume (Packard, Groningen, The Netherlands). The pellet containing the DNA was washed three to four times with PBS, resuspended in 200 μL Soluene (Packard) and counted in 10 mL Dimilume, for

* Abbreviations: dFdC, 2′,2′-difluorodeoxycytidine, (gemcitabine); ara-C, 1-β-D-arabinofuranosylcytosine; TdR, thymidine; UR, uridine; GIT, guanidine isothiocyanate; PBS, phosphate-buffered saline.

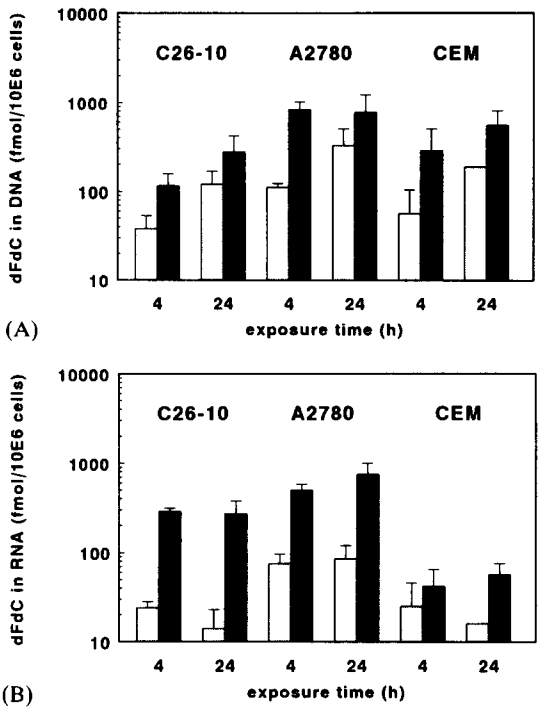

Fig. 1. Incorporation of dFdC into DNA (A) and RNA (B) of C26-10, A2780 and CCRF-CEM cells as measured by the acid precipitation assay. The values are means ± SEM of three to four experiments, except the 24-hr 0.1 μM DNA value of CEM cells. Hatched bars, exposure to 0.1 μM dFdC, closed bars to 1 μM. RNA content of these cell lines was 14.7, 14.8 and 1.8 μg/10^6 cells, respectively (means of two measurements). The RNA content of CCRF-CEM cells might be underestimated due to fragile nucleic acids, which are lost during purification.

5 min at a Kontron liquid scintillation counter. In order to determine DNA contamination of RNA, similar experiments were performed with [^{14}C]TdR (58.8 Ci/mol, 1.4 μM).

For the other assay we used a CsCl-gradient to separate the DNA and RNA. A minimum of 5–10 × 10^6 cells were exposed as described above. After counting, the cells were resuspended in 8 mL GIT buffer (4 M GIT, 0.12 mM β-mercaptoethanol in 25 mM sodium acetate in Milli Q sterile water, pH 6.0). The clear suspension was carefully layered on 4 mL CsCl (5.9 M CsCl in 25 mM sodium acetate in Milli Q sterile water, pH 6.0). The samples were centrifuged for at least 16 hr, in a Beckman SW-40 rotor at 32,000 rpm, at room temperature. The RNA pellet was resuspended in 300 μL of 0.3 M sodium acetate (pH 6.0) and counted for 5 min in 10 mL Highsafe (Packard). The interphase layer containing the DNA was removed and mixed with sterile distilled water upto 15 mL and subsequently 25 mL 95% ethanol were added. The DNA suspension was kept at −20° for 30 min. After centrifugation at 4000 *g* for 20 min the DNA pellet was resuspended in 1 mL water and counted for 5 min in 9 mL of Highsafe. Briefly, RNA was purified as follows. The RNA pellet was resuspended in 300 μL 0.3 M NaAc, pH 6.0. Then 300 μL SS-phenol and 300 μL chloroform were added, mixed and centrifuged. The upper aqueous layer was mixed with 1 mL of 95% ethanol. The mixture was kept at −20° for 15 min, followed by centrifugation at 12,000 *g*. The alcohol was discarded and another 1 mL of ethanol was added to the pellet. After 2 min centrifugation at 12,000 *g* the ethanol was carefully removed and the pellet was dried in a Savant Speedvac centrifuge. Before counting, the pellets were resuspended in water. With this purification all non-incorporated dFdC-associated radioactivity should be lost. Similar experiments were performed with [^{14}C]TdR, to detect contamination of the RNA.

Determination of DNA and RNA synthesis activity in whole cells. Cells were seeded at a density of 1 × 10^5 cells per well in a 96-well filtration plate, GVPP (Millipore Durapore) (Millipore B.V., Etten-Leur, the Netherlands), essentially as described previously [15, 16]. The cells were exposed to 0.1 and 1 μM dFdC for 4 and 24 hr (total volume 200 μL). Two hours before the end of the incubation, 10 μL of [^{14}C]TdR (58.8 Ci/mol, 567 pmol; DNA synthesis) or 10 μL of [^{3}H]UR (7.0 Ci/mmol, 198 pmol; RNA synthesis) were added. As a control untreated cells were incubated for 2 hr with [^{14}C]TdR or [^{3}H]UR. For a blank we added [^{14}C]TdR or [^{3}H]UR immediately before harvesting. For each experiment all incubations were performed in triplicate. At the end of the incubation period the plate was put on a vacuum manifold (Millipore), in order to filtrate the medium through the filters, followed by washing four times with 8% (w/v) ice-cold trichloro-acetic acid, four times with demi water and four times with 70% (v/v) ethanol. The bottom filters were dried by blowing cold air. The filters were punched out into scintillation vials. The [^{14}C]TdR samples could be counted directly after adding 5 mL of Optiphase 'HiSafe' III scintillation cocktail (LKB, FSA Laboratory Supplies, Loughborough, U.K.) in a LKB scintillation counter. The [^{3}H]UR filters required a 2 hr elution with 500 μL of 2 M NaOH. After adding 5 mL of Optiphase 'HiSafe' III scintillation cocktail and stabilization for 1 hr, radioactivity was counted.

RNA measurement in whole cells. Cell pellets were each suspended in 6 mL denaturation buffer [25 mM sodium citrate, 0.5% (w/v) *N*-laurylsarcosine and 0.12 mM β-mercaptoethanol in 4 M GIT]. Sodium acetate (0.6 mL, 2M, pH 4.0) was added and thoroughly mixed by inversion. Then 6 mL water-equilibrated phenol (pH 5.5–6.5) and 1.2 mL chloroform/isoamylalcohol (49/1) were added. After shaking well, the suspension was chilled on ice for 15 min, followed by centrifugation of 20 min, at 1800 *g* and 4°. To the upper (water) layer 6–8 mL of 2-propanol was added. After mixing well the mixture was put at −20° for 1 hr. After centrifugation for 20 min at 1800 *g* and 4°, the RNA pellet was dissolved in 1.8 mL denaturation buffer. A second precipitation was performed by 2-propanol, at −20° for 1 hr. The thus purified RNA pellet was resuspended in 270 μL water. An aliquot of 4 μL was taken for RNA estimation and diluted with water to 1 mL. The optical density was measured at 260 and 280 nm. The RNA was considered pure when the 260/280 ratio was 2 or higher [17].

Results

Measurement of dFdC incorporation using acid precipitation of nucleic acids revealed a clear time and concentration dependent incorporation into DNA in both A2780 and Colon 26-10 cells (Fig. 1A). These levels of incorporation (in A2870 3–5-fold higher than in C26-10) correlated with the sensitivity to dFdC of both cell lines. The IC_{50} for 24-hr exposure for A2780 was 2 ± 0.6 and for C26-10 22 ± 4 nM (mean ± SEM of three to four experiments) [10]. An unexpected, but clear incorporation of dFdC into RNA was observed. The incorporation was strongly concentration dependent. In A2780 cells the absolute incorporation was less than into DNA. In C26-10 cells, however, incorporation of dFdC into RNA was comparable to or higher than into DNA (Fig. 1B).

For comparison we also determined incorporation of dFdC into nucleic acids of CCRF-CEM cells, essentially according to the conditions used by Huang *et al.* [9] after exposure to 1 μM dFdC for 4- and 24-hr exposure.

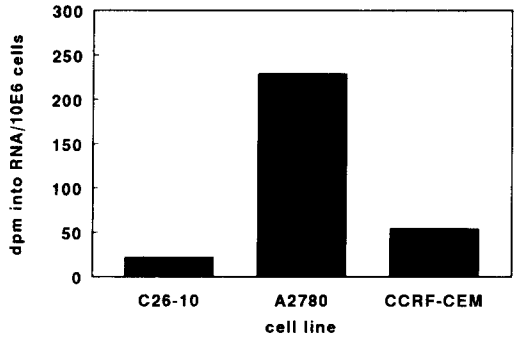

Fig. 2. Incorporation of dFdC into RNA of C26-10, A2780 and CCRF-CEM cells, using the semi-quantitative CsCl-gradient method in one typical experiment. Cells were exposed for 24 hr to 0.1 μM of dFdC. The number of cells used was at least 5.10^6 cells per sample. The values shown are corrected for the blanks.

Incorporation into DNA and RNA was also evident in this cell line. Incorporation into DNA was comparable to both solid tumour cell lines, but incorporation into RNA of the leukemic CCRF-CEM cells was much lower than in the A2780 and C26-10 cell lines, especially at 1 μM (Fig. 1).

In order to verify the incorporation of dFdC into RNA, we also separated both nucleic acids using semi-quantitative CsCl gradient centrifugation (Fig. 2). Incorporation into RNA of all three cell lines is evident using either method. To exclude the possibility of adhesion of dFdC to proteins surrounding RNA, we purified the isolated RNA of C26-10 and A2780 cells by phenol–chloroform extraction. The incorporation pattern was comparable to results without phenol–chloroform extraction (data not shown). DNA contamination of the RNA was determined to be 0.1–0.4% using [^{14}C]TdR incorporation under similar conditions as the dFdC incubation in both assays. Incorporation of dFdC into RNA exceeded this contamination (10–250-fold, depending on the cell line).

In all three cell lines dFdC almost completely inhibited DNA synthesis. This inhibition was already observed at 4-hr exposure to 0.1 μM dFdC and was most pronounced in CCRF-CEM and A2780 cells (Fig. 3). RNA synthesis was inhibited too, but to a lesser extent than the DNA synthesis. In the two solid tumour cell lines the inhibition of RNA synthesis varied between 10 and 40%. In C26-10 cells inhibition was least evident. In the leukemic cell line CCRF-CEM inhibition of RNA synthesis was clearly time dependent. At 4-hr exposure to dFdC no significant inhibition was observed, at 24-hr exposure 1 μM of dFdC inhibited RNA synthesis completely.

At 4-hr dFdC exposure, when no significant inhibition of RNA synthesis was observed, dFdC incorporation at 1 μM dFdC into RNA was 3.7, 1.4 and 0.2% of UR incorporation for C26-10, A2780 and CCRF-CEM cells, respectively. The initial incorporation of dFdC into DNA during exposure to 1 μM of dFdC was estimated to be at least 0.04, 0.3 and 0.08% of that of thymidine for C26-10, A2780 and CCRF-CEM cells, respectively. Due to the very rapid and almost complete inhibition of DNA synthesis these values are underestimated.

Discussion

In this study we demonstrate a clear concentration-dependent incorporation of dFdC into RNA as well as a time- and concentration-dependent incorporation into DNA, in addition to a pronounced inhibition of both RNA and DNA synthesis by dFdC. These results are partly in contrast to previous studies [9], in which neither

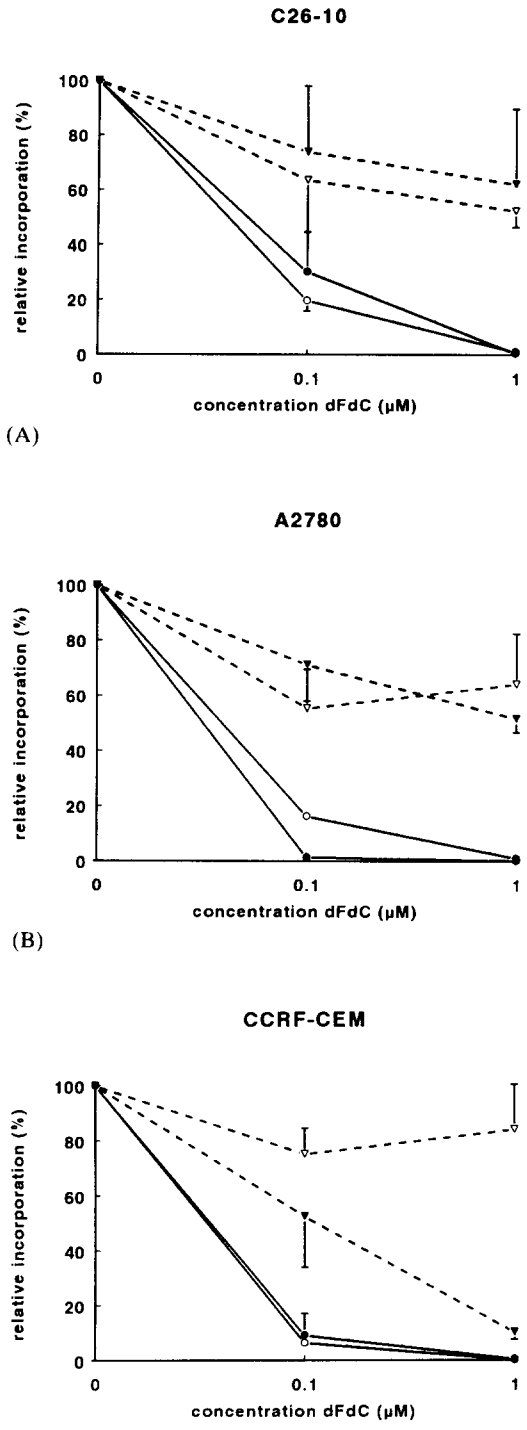

Fig. 3. Inhibition of DNA and RNA synthesis as determined in C26-10 (A), A2780 (B) and CCRF-CEM (C) cells. The open triangles represent RNA synthesis at 4-hr dFdC exposure; the closed triangles RNA synthesis at 24-hr, the open circles DNA synthesis at 4-hr and the closed circles DNA synthesis at 24-hr exposure. Values are means of three to four experiments ± SEM. The absolute TdR incorporation into DNA of untreated C26-10, A2780 and CCRF-CEM cells was 65, 60 and 91 pmol/hr/10^6 cells, respectively. Absolute UR incorporation into RNA of untreated cells was 2.0, 8.6 and 5.7 pmol/hr/10^6 cells, in C26-10, A2780 and CCRF-CEM, respectively.

incorporation into RNA of CCRF-CEM cells nor inhibition of RNA synthesis was observed after a 4-hr exposure to 1 μM dFdC. We determined dFdC incorporation using two assays, both of which obtain the RNA in one sample to be counted. Huang *et al.* [9] used Cs_2SO_4 gradient centrifugation, after which the RNA was collected in several fractions; the radioactivity present in the isolated RNA might be diluted too extensively in order to be detected in the separate fractions. Although not all conditions used by Huang *et al.* [9] were described extensively, it might be that the conditions were not optimal for detection of the relatively low amount of radioactivity incorporated into RNA of the CCRF-CEM cells. As to the RNA synthesis, our results confirm those of Huang *et al.* [9]. At a 4-hr exposure to 1 μM of dFdC no inhibition was observed, when the CCRF-CEM cells were incubated for 24 hr with 1 μM of dFdC, however, RNA synthesis was inhibited completely (Fig. 3).

It is unlikely that the amount of radioactivity found in the RNA is due to artefacts of the assays. In the acid precipitation assay we used DNase free RNase, while the pellets were washed thoroughly. Contamination of RNA with DNA was less than 0.4%, as measured by [^{14}C]TdR incorporation under similar conditions. The RNA pellet isolated from the CsCl gradient also contained less than 0.4% contamination from DNA. It is unlikely that radioactivity is aspecifically bound to proteins associated with RNA, since this would be removed during the further extensive purification of the RNA. The detection limit for RNA incorporation was 1.3 fmol/10^6 cells for the acid precipitation assay and 0.7 fmol/10^6 cells for the CsCl method.

Comparison of dFdC incorporation into DNA with normal DNA synthesis, demonstrated that the estimated initial dFdC incorporation in the solid tumour cell lines correlates with the sensitivity of those cells to dFdC. As to the incorporation into RNA, C26-10 showed the highest relative dFdC incorporation, but A2780 cells the highest absolute incorporation.

The significance of the incorporation into RNA is not yet clear. For another cytidine analogue, ara-C, the effect on nucleic acids is limited to incorporation into DNA [18, 19]. This remarkable difference between ara-C and dFdC makes the latter compound a clearly different antimetabolite. A clear relationship between the extent of dFdC incorporation into DNA and sensitivity to dFdC has been demonstrated for several cell lines [7–9], including this study, in which the more sensitive A2780 showed a higher incorporation into DNA than the more resistant C26-10. The two solid tumour cell lines showed a considerably higher amount of incorporation into RNA than the leukemic cell line. It is not yet clear whether the RNA incorporation will contribute much to the direct anti-tumour effect. However, the interference with RNA might lead to delayed toxic side effects *in vivo*. Although this concept has still to be proven, this offers the possibility for selective modulation of the activity of dFdC, similarly to that of 5-fluorouracil [20]. Therefore, future studies will focus on the mechanism of RNA incorporation, determining the exact metabolite which is incorporated into RNA (dFdCTP, dFdUTP). In addition, the effect of dFdC on RNA synthesis and functioning require further in depth study.

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