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SHORT COMMUNICATIONS

Activation and cytotoxicity of 5′-deoxy-5-fluorouridine in c-H-*ras* transformed NIH 3T3 cells

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Colorectal cancer is the second leading cause of cancer death in United States [1]. Extensive studies on chemotherapeutic and radiotherapeutic approaches to the treatment of the disease have not resulted in any significant progress thus far.

Most of nowadays anticancer agents fail to induce clear responses in the treatment of colorectal cancer. This might be related: (i) with detoxification mechanisms of intestinal cells [2]; (ii) with *ras* gene mutations, which have been demonstrated frequently in human colorectal cancers and were found to be associated with antitumour drug resistance [3, 4], and (iii) with overexpression of P-glycoprotein (Pgp), a superfamily of membrane-linked transport proteins associated with multidrug resistance [5].

Interestingly, fluoropyrimidines are amongst the few options in the chemotherapeutic treatment of colorectal cancers (Fig. 1).

The latest fluoropyrimidine 5′-deoxy-5-fluorouridine (dFUrd*) proved to be at least compatible with FUra and FdUrd in the treatment of colorectal cancer [6]. One of the key-role enzymes of dFUrd activation is pyrimidine nucleoside phosphorylase which converts dFUrd into FUra [6].

Tumour cells frequently exhibit increased pyrimidine nucleoside phosphorylase activity and therefore dFUrd exposure results in targeted FUra release in tumour cells (Fig. 2) [6].

In the present study we explored the possibility of *ras* involvement in dFUrd activation in c-H-*ras* transformed NIH 3T3 cells. We found that cells transformed by c-H-*ras* were significantly more sensitive to dFUrd owing to increased intracellular FUra production. If these findings should prove to apply to *ras* involvement specifically, the presence of activated *ras* genes might help to select patients for dFUrd treatment.

Materials and Methods

Drugs and chemicals. dFUrd, FUra and $FUraH_2$ were kindly supplied by Hoffmann-La Roche (Mijdrecht, The Netherlands). Diphenylsuccinimide was synthesized by Chemische Industrie Katwijk (Katwijk, The Netherlands) and purified by recrystallization from tetrahydrofuran before use. 5-Chlorouracil was purchased from Calbiochem (Los Angeles, CA, U.S.A.). 2-Thiouracil was obtained from Merck (Darmstadt, F.R.G.). All solvents were of analytical grade and used as received from Baker Chemicals (Deventer, The Netherlands). dFUrd was supplied in sterilized flasks each containing 1 g lyophilized drug, which was dissolved in sterile water to a maximum final concentration of 100 mg/mL.

Cell lines. NIH 3T3 cells were transformed by transfection with molecularly cloned c-H-*ras*. Data of clonogenic assays and incubation tests with the transformed cells were compared with control NIH 3T3 cells. Transformed cells were cloned and tested for the ability to grow in soft agar and take rate studies in nude mice. Transformed cells were tested for the presence of the transfected gene by Southern blot analysis [7] using a H-*ras* probe.

Clonogenic assay/dFUrd and FUra cytotoxicity. Cells in exponential growth in Dulbecco's medium with glutamin and pyruvate but without antibiotics, and 10% foetal bovine serum (D10 medium) were exposed to concentrations and exposure times according to physiologic circumstances [8]: 1–100 μg/mL with exposure time $t = 1$ hr.

After removal of the agents of interest by triple washing with phosphate-buffered saline, the cells were treated with a 0.1% trypsin solution, counted (by Coulter Counter), and replated in triplicate. The plates were fixed and stained with 1% Methylene blue in 70% methanol after approximately 8 days and colonies were scored. A colony was defined as a group of more than 30 cells.

Per cent survival was calculated by dividing the mean number of colonies in the treated sample by mean number in the coherent control (untreated) sample and multiplying by 100. The surviving fractions of cells plated was plotted against the concentration of dFUrd and FUra in the medium.

The concentration of a drug at which colony formation is 50% inhibited as compared to colony formation of untreated cells (ID_{50}) was used as parameter of sensitivity

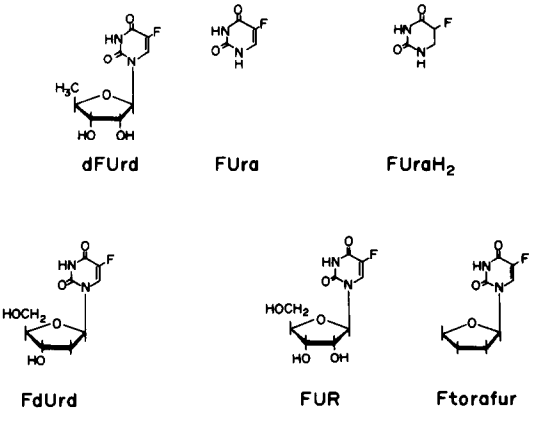

Fig. 1. Structures of fluorouracil (FUra); 5-fluoro-2′-deoxyuridine (FdUrd); 5-fluorouridine (FUR) and 5′-deoxy-5-fluorouridine (dFUrd). Furthermore the structure of 5,6-dihydrofluorouracil ($FUraH_2$), the first product of FUra catabolism, is indicated.

* Abbreviations used are: dFUrd, 5′-deoxy-5-fluorouridine; FUra, 5-fluorouracil; FdUrd, 5-fluoro-2′-deoxyuridine; FUrd (or FUR), 5-fluorouridine; FUMP, 5-fluorouridine-5′-monophosphate; FUDP, 5-fluorouridine-5′-diphosphate; FUTP, 5-fluorouridine-5′-triphosphate; FdUrd, 5-fluoro-2′-deoxyuridine; FDUMP, 5-fluoro-2′-deoxyuridine-5′-monophosphate; FdUDP, 5-fluoro-2′-deoxyuridine-5′-diphosphate; FdUTP, 5-fluoro-2′-deoxyuridine-5′-triphosphate; HPLC, high performance liquid chromatography; CGC, capillary gas chromatography; TS, thymidylate syntase.

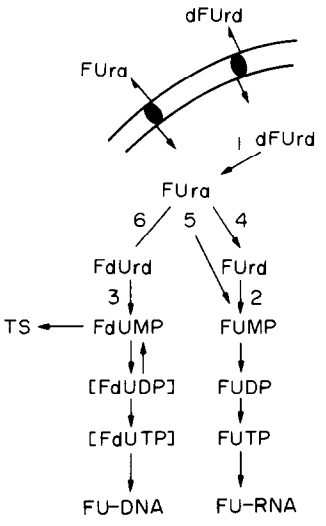

Fig. 2. Activation of dFUrd (5'-deoxy-5-fluorouridine) and further metabolism of either intracellularly formed FUra (5-fluorouracil) or transported FUra which is produced following extratumoural dFUrd → FUra activation. Enzymes involved are: 1,pyrimidine nucleoside phosphorylase; 2, uridine kinase; 3, thymidine kinase; 4, uridine phosphorylase; 5, orotate phosphoribosyltransferase and 6, thymidine phosphorylase. FUra and subsequently $FUraH_2$ can be formed in the liver and released to the systemic circulation, therefore tumour cells are exposed to both dFUrd and FUra [6].

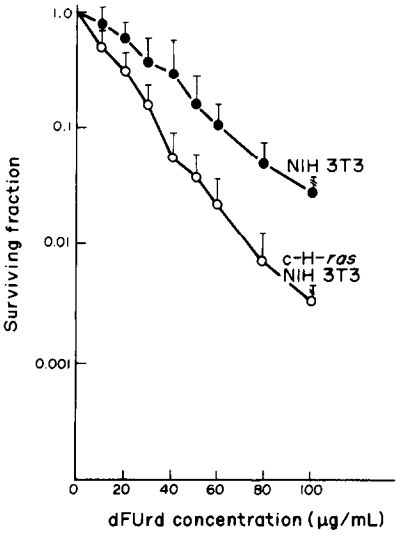

Fig. 3. Significantly increased dFUrd sensitivity in c-H-*ras* transformed cells as determined by clonogenic assay. The surviving fraction of cells plated was plotted against the concentration of dFUrd. Surviving fractions vs dFUrd concentration (mean ± 2 SD; N = 3) are indicated.

for dFUrd and FUra. The *RF* is the ratio of the ID_{50} of the NIH 3T3 line and the ID_{50} of the transformed NIH 3T3 line. Data analysis was performed by Student's two sample *t*-test, with $P < 0.05$ as the level of significance.

Incubation with dFUrd. A fresh solution of the i.v. formulation of dFUrd was prepared in 0.85% saline solution. Transformed and control cells were incubated with dFUrd (1 μg/mL) in Hank's Balanced Salt Solution and 10% foetal calf serum, while shaking in a water bath during 1 hr. For each experiment (N = 6) NIH 3T3 control and transformed cells were used.

After drug exposure and centrifugation the drug containing media were decanted and subjected to dFUrd, FUra and $FUraH_2$ determination [8, 9]. For comparisons between the capacity of dFUrd conversion *in vitro*, the Student's *t*-test for unpaired samples was used.

Results

The transformed cell line was significantly more sensitive to dFUrd than NIH 3T3 cells at all concentrations of dFUrd tested as shown in Fig. 3 ($P < 0.01$).

The *RF* was 2.4. The transformed cell line was also more sensitive to FUra; however, the difference between transformed cells and NIH 3T3 cells failed to reach significance ($0.05 < P < 0.1$, data not shown).

The difference between sensitivities of both cell lines to dFUrd was demonstrated to be related with increased dFUrd activation *in vitro*, which is known to be mediated by pyrimidine nucleoside phosphorylase [6]. Recovery of FUra following 1 hr incubation of NIH 3T3 cells with dFUrd was <5%. Transformed cells demonstrated a significantly higher ($P < 0.05$) FUra recovery: 56.3–81.9%. $FUraH_2$ could not be demonstrated and therefore FUra catabolism during incubation was excluded.

Discussion

We have now shown that dFUrd activation in NIH 3T3 cells can be increased significantly upon transformation with c-H-*ras*: the transformed cells are more sensitive to dFUrd owing to increased intracellular FUra production. This might have consequences for dFUrd activity *in vivo*. *ras* genes are highly conserved in mammalian cells and should these findings also prove to apply to *in vivo* situation including tumours with *ras* involvement, the presence of activated *ras* genes might help to predict clinical response to dFUrd and related fluoropyrimidines.

Transformed NIH 3T3 cells can generate tumours in nude mice and the hypothesis could therefore be tested *in vivo* using c-H-*ras* transformed cells versus a cell line transformed by an unrelated oncogene (e.g. v-*fos*).

In nude mice bearing tumours of NIH 3T3 cells transformed with c-H-*ras*, dFUrd was capable of inducing tumour remission upon continuous (10 days) low dose treatment. Remarkably, in v-*fos* transformed tumours remission could not be induced upon the same treatment. These findings need to be established with experiments including more *ras* unrelated oncogenes (e.g. v.-*fms*, v-*mos*) as well as several types of cellular and viral *ras* oncogenes and a normal *ras* protooncogene activated by over-production driven by an mLV *ltr*, in order to define the precise role of *ras* in dFUrd cytotoxicity [4, 10].

Nevertheless, the data of the present study are of great importance from both a fundamental and clinical point of view.

Increased dFUrd is most likely associated with increased pyrimidine nucleoside phosphorylase activity and it can be questioned how the enzyme is (in)directly governed by transformation with c-H-*ras*. Ongoing experiments with other oncogene transformed cells mentioned above, together with measurement of pyrimidine nucleoside phosphorylase activity at the level of transciption and enzymatic level, will reveal intrinsic interaction between oncogene transformation and activity of the enzyme.

We were able to establish the consequences of increased dFUrd activation in tumour-bearing mice. This is important with respect to the clinical situation: fluoropyrimidines are amongst the few chemotherapeutic options in the treatment of colorectal cancer, most likely because: (i) the activity is

not mediated by known detoxification mechanisms [2]; (ii) the activity is not affected by the multidrug resistance family of proteins with a molecular weight of approximately 170,000 [5]; and (iii) formation of cytotoxic metabolites of FUra such as FdUMP was at least not decreased upon *ras* transformation since cytotoxicity of FUra was the same or even not significantly increased in c-H-*ras* transformed and control cells. It is not yet known whether this newly demonstrated dFUrd activity enhancement can be exploited optimally in c-H-*ras* transformed cells.

It is frequently stated that continuous dFUrd treatment might be more effective than bolus dFUrd [6]. Nude mice bearing NIH 3T3 cells transformed with *ras* and other oncogenes as discussed above offer an interesting model to define optimal treatment with dFUrd. This is especially true for colorectal tumours since *ras* involvement was frequently demonstrated in this tumour type [3] and dFUrd has proven efficacy in the treatment of this tumour. Since activated *ras* oncogenes have also been associated with resistance to cisplatin and to ionizing radiation [4, 10], the present data add to the understanding of the rather unique efficacy of fluoropyrimidines in the treatment of colorectal cancer.

In summary, transformation of NIH 3T3 cells with c-H-*ras* has been demonstrated to result in significantly increased activation of 5'-deoxy-5-fluorouridine and significantly increased cytotoxicity *in vitro* as compared to non-transformed NIH 3T3. FUra cytotoxicity appeared to be increased also *in vitro* upon transformation; the level of significance however was beyond that of accepted significance ($0.05 < P < 0.01$). Furthermore dFUrd proved to be less active *in vivo* in nude mice bearing v-*fos* transformed NIH 3T3 cells than in nude mice bearing c-H-*ras* transformed cells.

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Hyposmolarity-sensitive release of taurine and free amino acids from human lymphocytes

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The ability of human lymphocytes to regulate their volume in anisosmotic conditions is well documented [for recent reviews see Refs. 1 and 2]. When exposed to hyposmotic media, human lymphocytes show rapid initial swelling followed by a regulatory phase in which cells return to near normal volume. The regulatory volume decrease following osmotic swelling results from the loss of intracellular osmotically active solutes, mainly K^+ and Cl^-. In human lymphocytes these ionic regulatory fluxes occur through separate K^+ and Cl^- pathways [1, 3, 4], whereas in other cells they are carried by K^+/Cl^- cotransport systems, activated by the osmotic stress [5].

Although the reduction in the amount of intracellular solutes leading to cell volume regulation corresponds largely to the loss of K^+ and Cl^-, some amino compounds also behave as intracellular osmolytes and may contribute at some extent to the regulatory process. The involvement of free amino acids (FAA) in osmoregulation in aquatic vertebrates and invertebrates naturally exposed to fluctuations in external osmolarity is well recognized [6, 7].