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A Non-Radioactive Sensitive Assay to Measure 5-Fluorouracil Incorporation into DNA of Solid Tumors

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ABSTRACT

A non-radioactive method to determine 5-Fluorouracil (5FU) incorporation into DNA has been developed. Isolated DNA was enzymatically degraded to bases and the resulting 5FU was measured with standard gas-chromatography coupled to mass spectrometry (GC-MS) and compared with that of radioactive 5FU in a cell line. Incorporation into DNA of the murine Colon 26-B tumor treated with maximal tolerated doses of 5FU and fluorodeoxyuridine (FUDR) was maximal after 2 hour and was 15.4 and 71.0 fmol/μg DNA, respectively. After a plateau for about 3 days a decrease was observed to ± 2 fmol/μg DNA after 10 days. The assay is very sensitive and reproducible and can be used in a clinical setting.

Key Words: 5-Fluorouracil; RNA incorporation and DNA incorporation.

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INTRODUCTION

The mechanism of action of 5FU has been related to inhibition of thymidylate synthase (TS)^[1] while the incorporation into RNA and DNA may also play a role in the efficacy of the drug.^[2] Inhibition of TS results in depletion of dTTP and an increase of dUTP followed by a decreased DNA synthesis. Addition of Leucovorin which acts as precursor of 5,10-methylene-tetrahydrofolate, and is necessary for the formation of the ternary complex with TS and FdUMP, potentiates the inhibition of TS. Incorporation of 5FU into RNA may lead to disturbance of mRNA processing and protein synthesis but could be decreased with UDP-glucose without affecting the efficacy of the drug,^[3] indicating that incorporation into RNA only plays a minor role in the anti tumor activity. 5FU incorporation into DNA may also play a role in the cytotoxic effect of the drug,^[4] due to the formation of DNA strand breaks after excision by uracil DNA glycosylase. Repair of these strand breaks is hampered by the imbalance of the deoxynucleotide pools as result of TS inhibition. Incorporation of FdUTP and dUTP into DNA is dependent on the action of dUTPase.^[5] Since the use of radio labeled 5FU is not practicable in a clinical setting, we developed a non-radioactive assay to determine the 5FU incorporation into DNA.

METHODS

Isolated and purified DNA from murine WiDr colon cancer cells and Colon26-B tumor tissue was dissolved in digestion buffer containing 40 mM Tris, 1 mM MgCl₂, 0.1 mM ZnCl₂ and 40 mM KH₂PO₄, pH=7.4 at 55°C. To 340 µl DNA suspension 20 µl DNase I (1 mg/ml), 20 µl Nuclease P1 (250 U/ml), 10 µl Alkaline Phosphatase (1000 U/ml) and 10 µl Thymidine Phosphorylase (600 U/ml) were added. Incubation of this reaction mixture overnight at 37°C resulted in complete degradation of the DNA to bases, including 5FU. After addition of 50 µl of the internal standard 5FU-¹⁵N₂ (0.1 µM), 5FU was measured after extraction and derivatization with pentafluorobenzyl-bromide. GC-MS measurement of 5FU was described before.^[6]

RESULTS

The assay was first validated with WiDr Colon cancer cells as described for 5FU incorporation into RNA.^[6] The incorporation of 25 µM 5FU into DNA after 2 hr and 4 hr was 0.15 ± 0.02 and 0.35 ± 0.09 pmol/µg DNA as determined with GC-MS (means \pm SEM). At 50 µM the 5FU incorporation after 2 and 4 hr was 0.35 ± 0.097 and 0.57 ± 0.141 pmol/µg DNA, respectively. This was in the same range as described previously with radioactive 5FU in this cell line, 0.63 ± 0.11 pmol/hr/10⁶ cells at 15 µM 5FU.^[7] Subsequently incorporation into DNA of Colon26-B tumors was determined. Incorporation into DNA after treatment with 5FU (80 mg/kg) or FUDR (400 mg/kg) was maximal at 2 hours after drug treatment and was 15.4 and 71.0 fmol/µg DNA, respectively (Fig. 1). The level of incorporation remained high for about 3 days after which a decrease was observed to ± 2 fmol/µg DNA after 10 days both for

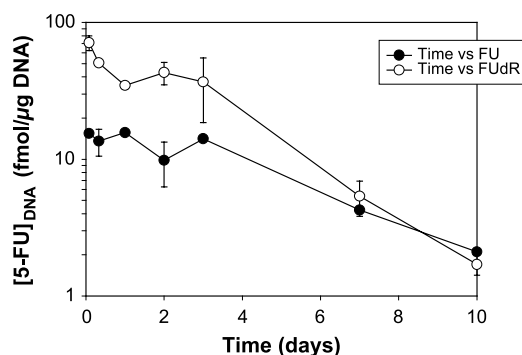


Figure 1. Incorporation of 5-FU into DNA of murine Colon-26B tumor tissue at different time points after administration of 80 mg/kg 5-FU (closed circles) and 400 mg/kg FdR (open circles). Values are means of 2–3 samples \pm SD.

5FU and FdR. In contrast to RNA, DNA incorporation did not correlate with the intra-tumoral concentration of 5FU, neither for 5FU or FdR.^[8]

DISCUSSION

The higher initial incorporation into DNA is in agreement with a higher formation of FdUMP from FdR compared to 5FU.^[8] Apparently potential breakdown of FdUTP by dUTPase did not prevent the incorporation into DNA. The relatively long retention of 5FU in DNA might be due to the favorable incorporation conditions because of dTTP depletion as result of TS inhibition but also to a rather inefficient repair by Uracil DNA Glycosylase. The data give strong evidence that DNA incorporation of fluoropyrimidines contributes to their antitumor activity since the better antitumor activity of FdR was associated with a higher extent of incorporation of FdR into

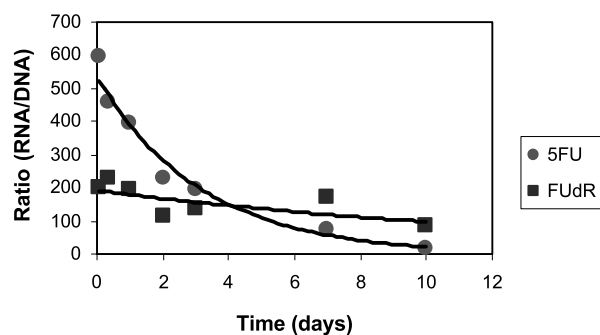


Figure 2. Ratio of incorporation of fluoropyrimidines into RNA and DNA. For each separate tumor the incorporation into DNA and RNA after treatment with 5FU or FdR was calculated and plotted as (pmol/μgRNA)/(fmol/μgDNA). The RNA data have been published earlier. (From Ref. [8].)

DNA as compared to 5FU. When incorporation into RNA and DNA are compared by calculation of the ratio RNA/DNA, a low ratio is found for FUDR treatment compared to 5FU treatment (Fig. 2). This ratio is 3-fold higher for 5FU treatment, but the antitumor effect is less than that of FUDR,^[8] underlining that incorporation into RNA does not (or minimally) contribute to the antitumor activity of fluoropyrimidines.

This method will be used to evaluate the role of 5FU incorporation into DNA in tumor tissue during treatment of patients and the relative role of 5FU incorporation into DNA compared to RNA incorporation and the inhibition of TS. Furthermore this approach may help to evaluate potential changes in the mechanisms when 5FU is combined with other drugs such as irinotecan, oxaliplatin and raltitrexed.

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