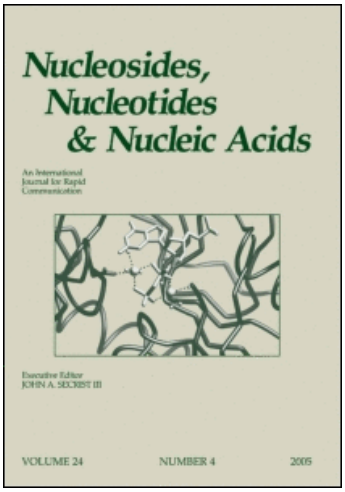

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Selective Protection by Uridine of Growth Inhibition by 5-Fluorouracil (5FU) Mediated by 5FU Incorporation into RNA, But Not the Thymidylate Synthase Mediated Growth Inhibition by 5FU-Leucovorin

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To cite this Article Codacci-Pisanelli, G. , Noordhuis, P. , van der Wilt, C. L. and Peters, G. J.(2008) 'Selective Protection by Uridine of Growth Inhibition by 5-Fluorouracil (5FU) Mediated by 5FU Incorporation into RNA, But Not the Thymidylate Synthase Mediated Growth Inhibition by 5FU-Leucovorin', Nucleosides, Nucleotides and Nucleic Acids, 27: 6, 733 — 739
To link to this Article: DOI: 10.1080/15257770802145496
URL: http://dx.doi.org/10.1080/15257770802145496

Nucleosides, Nucleotides, and Nucleic Acids, 27:733–739, 2008

ISSN: 1525-7770 print / 1532-2335 online
DOI: 10.1080/15257770802145496

Taylor & Francis
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SELECTIVE PROTECTION BY URIDINE OF GROWTH INHIBITION BY 5-FLUOROURACIL (5FU) MEDIATED BY 5FU INCORPORATION INTO RNA, BUT NOT THE THYMIDYLATE SYNTHASE MEDIATED GROWTH INHIBITION BY 5FU-LEUCOVORIN

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□ *Fluorouracil (5FU) acts by RNA-incorporation and inhibition of thymidylate synthase; the first action is counteracted by uridine, and the second is enhanced by leucovorin (LV). Growth inhibition of C26-10 colon cancer cells by 5FU was enhanced by LV and rescued by uridine, but 5FU-LV was only partially rescued by uridine. In WiDr cells, 5FU sensitivity was not enhanced by LV, while both 5FU and 5FU-LV were rescued by uridine. Intermediate trends were found in SW948 and HT29 cells. Uridine rescue in mice allowed 1.5-fold increase in 5FU dose, leading to 2-fold increase in the antitumor effect and thymidylate synthase inhibition in resistant Colon-26 tumors. In the sensitive Colon-26-10 tumor, uridine rescue decreased 5FU-RNA incorporation > 10-fold, without affecting the antitumor activity. The use of LV and uridine can differentiate between two mechanisms of action of 5FU.*

Keywords 5-fluorouracil; thymidylate synthase; 5FU incorporation into RNA; uridine protection

INTRODUCTION

Fluorouracil (5FU) has multiple possible mechanisms of interference with the proliferative activity of cells (Figure 1): Its anabolites can block thymidylate synthase (TS),[1] can interfere with RNA synthesis[2] and it can also be incorporated into DNA.[3,4] RNA interference and TS inhibition represent the main cellular targets, but the relative importance of each varies according to the characteristics of the cell line tested.

The identification of the main target is not trivial: the knowledge of 5FU intracellular metabolism allowed the use of biochemical modulation in order to funnel the drug preferentially toward one target while sparing

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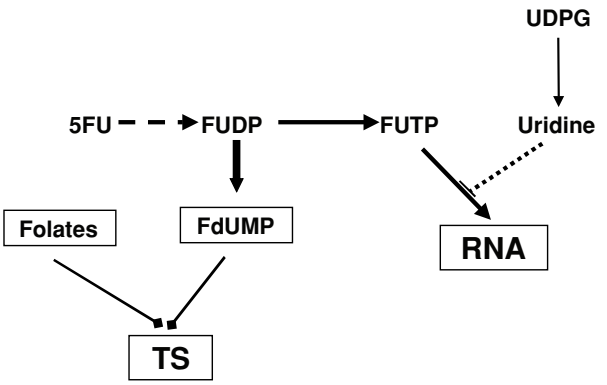

FIGURE 1 5-Fluorouracil (5FU) needs several activation steps to FUDP, after which it can interfere with cell proliferation through two main mechanisms: FUTP can be incorporated into RNA and FdUMP can inhibit TS. 5FU can be modulated using folates (specifically LV) (increased TS inhibition) and Urd (decrease of RNA incorporation). In animals Urd can also be given as UDP-glucose (UDPG) which acts as a prodrug for Urd.

the other.[5] This has been particularly successful with the use of Leucovorin (LV) as a potentiating agent, but it has also been attained with uridine (Urd) or its pro-drugs as a protecting agent.[5]

The aim of the present work was to identify the relative importance of RNA and TS as targets of the antiproliferative activity of 5FU by using Urd and LV as modulating agents.

MATERIALS AND METHODS

Four colon cancer cell lines were used: the murine C26-10 (relatively sensitive to 5FU) and the human WiDr, SW948 and HT29. Cells were grown in RPMI with 10% FCS.[6,7] Cells were exposed for 24 hours to varying concentrations of 5FU and this was followed by rescuing with Urd for 48 hours. 5FU concentrations that caused a 80—90% growth reduction when used alone were chosen for each cell line: 1, 5, 25, or 50 μM. LV (10 μM) was added concomitantly with 5FU.[6] Uridine (100 μM) was added after 24 hours and maintained for 48 hours. In control cells normal medium was added at this time. Cell proliferation was measured by counting the cells after trypsinization using an electronic cell counter.[8] Results are expressed as the percentage of growth relative to untreated controls, which were set at 100%, while the number of cells at the time of 5FU addition was set at 0%.

Animal experiments: Balb/c mice bearing Colon 26-10 or Colon 26 tumours implanted subcutaneously were treated weekly with 5FU at the maximum tolerated dose. Treatment was combined with LV and was followed by rescue with UDPG.[9]

The enzyme activity of TS was evaluated with a radioactive assay in which ^3H_2O was released from ^{3}H-dUMP.[6,10]

RESULTS

C26-10 cells: In this cell line 1 μM 5FU inhibited growth to 13% and could be rescued by Urd, 5FU activity was increased by LV and relative growth was 1%, but this could still be rescued by Urd to an almost normal value (83%; Figure 2). 5 μM 5FU caused complete growth inhibition that could not be rescued by Urd (not shown).

WiDr cells: The lowest concentration of 5FU used was 5 μM with a relative growth of 22% (Figure 2). This was not potentiated by LV (relative growth 19%). In both cases Urd could effectively rescue cells from 5FU damage (67% after 5FU alone, 72% after 5FU-LV). When a higher 5FU concentration was used (25 μM) the relative growth was 10%, and Urd had only a limited rescuing effect on the cells (relative growth 31%).

SW948 and HT29 cells: In SW948 cells the antiproliferative activity of 5FU at 5 μM was not affected by LV addition, (Figure 2). 5FU alone was completely rescued by Urd (105%); the activity of 5FU-LV was only partially reduced by Urd (75%). Similar results were found in HT29 cells for the combination of 5FU-Urd and 5FU-LV (not shown). At higher concentrations of 5FU (up to 100 μM), growth inhibition was usually complete, could not be enhanced by LV or rescued by Urd. These concentrations are outside the range of commonly found 5FU plasma levels.[5]

The sensitive C26-10 cells were also evaluated in vivo as the tumor Colon 26-10, together with the resistant variant Colon 26. The maximum tolerated dose of weekly 5FU could be increased from 100 to 150 mg/kg with Urd rescue (given as UDP-glucose)[9]. In Colon 26 and in Colon 26-10 TS inhibition was 2- to 3-fold increased (at day 3 and 10, each 3 days after a 5FU injection, at day 0 and 7, respectively) at the higher dose and associated with a 2-fold increased antitumor effect in Colon 26. In Colon 26-10, Urd rescue decreased incorporation of 5FU into RNA >10-fold and allowed a faster removal of FUTP from RNA (Figure 3), but did not affect the antitumor effect or TS inhibition.

DISCUSSION

The knowledge of the intracellular activation of 5FU allowed a more rational use of this drug through modulation. This also makes it possible to design experiments that may help to easily identify the role of the different mechanisms of action of this molecule. The interference with RNA is blocked by Urd rescue, while TS inhibition is improved by LV coadministration.

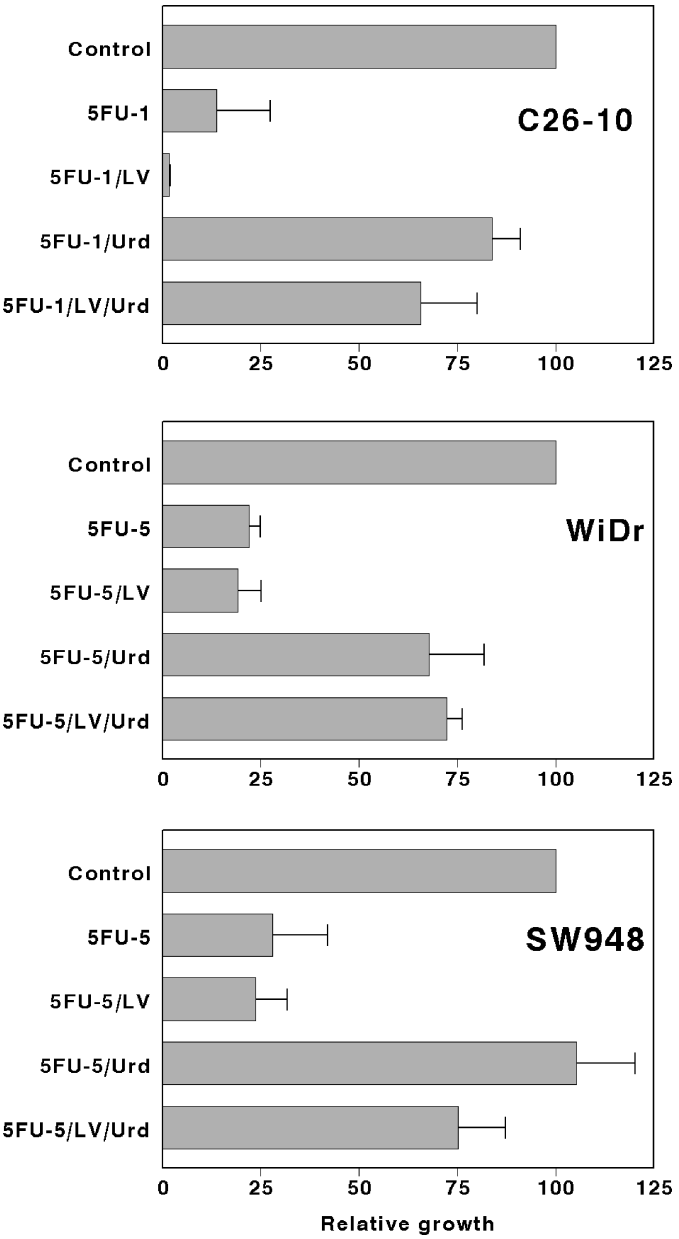

FIGURE 2 Modulation of colon cancer cells by LV and uridine. 5FU-1 and 5FU-5 indicate addition of 5FU at 1 or 5 μM. LV was added simultaneously with 5FU. Urd (uridine) was added after 24 hours, after washing the cells. Values are means ± SE of at least three separate experiments.

In all cell lines the growth inhibition by low 5FU concentrations could (partially) be rescued by Urd, while in three cell lines (C26-10, SW948, and HT29) the sensitivity to low concentrations of 5FU could be enhanced by LV, although to a lesser extent in SW948 and HT29 compared to C26-10.

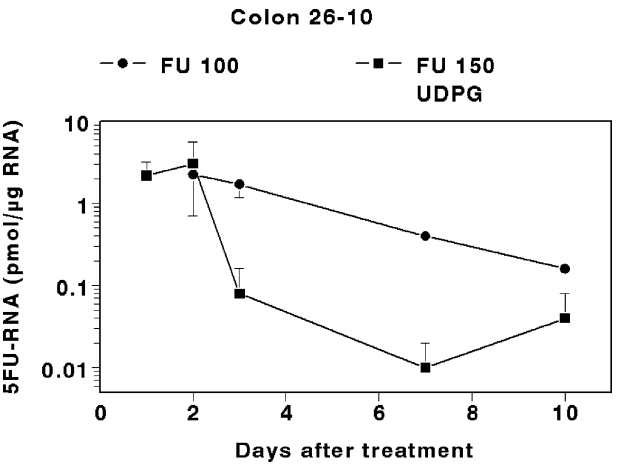

FIGURE 3 Incorporation of 5FU into RNA from sensitive Colon 26-10 implanted in mice treated with 5FU at the maximal tolerated dose for single administration (100 mg/m^2) or in combination with Urd (5FU at 150 mg/m^2). Tumors were snapfrozen in liquid nitrogen, pulverized and RNA was extracted, and degraded to 5FU, which was measured with gas-chromatography coupled to mass-spectrometry. Despite the higher dose of 5FU in combination with Urd, incorporation into RNA was decreased compared to the lower dose of 5FU. Values are means ± SD from at least 3 mice (6 tumors) for each data point (modified from 8).

This indicates that the mechanism of action of 5FU could be shifted by LV to inhibition of TS. However, at higher 5FU concentrations, LV did not potentiate anymore, indicating that TS inhibition is complete and irreversible. The selective effect of LV on 5FU could also be concluded out of the lack of (or only partial) rescue by Urd of the LV-5FU combination. At higher 5FU concentrations the growth inhibition could not be rescued by Urd. We can assume that in these conditions TS inhibition has a more relevant role and cells cannot be rescued by reducing the RNA effect.

The selective modulation also showed that in WiDr cells the mechanism of action of 5FU is predominantly mediated by its incorporation into RNA, since it could not be potentiated by LV. By using either radioactive 5FU or sensitive gas-chromatography coupled to mass spectrometry, it was demonstrated that 5FU can be incorporated to a high extent into RNA of these cells,[8,11,12] which could be reversed by Urd.[8] In WiDr cells recovery from TS inhibition was relatively rapid compared to other cell lines.[11]

The most convincing evidence for the selective protective effect of Urd on 5FU toxicity was observed in vivo. Urd protected against toxic side effects of bolus 5FU, allowing to increase the dose of 5FU by 150%, thereby increasing the retention of TS inhibition and reducing the incorporation into RNA, without affecting the antitumor activity.[9,10] This also demonstrated that not only a continuous infusion or prolonged exposure,[13] but also a bolus injection of 5FU leads to TS inhibition. In addition,

LV enhanced the extent and retention of inhibition of TS.[10] The latter is mediated by the formation of a stable ternary complex between TS, FdUMP and 5,10-methylenetetrahydrofolate, for which LV is the direct precursor.[5] The stability of the complex, and hence the retention of TS inhibition, is enhanced by providing extracellular folates in the form of LV. We recently demonstrated that these findings can be extrapolated to the clinic, in which we not only demonstrated that response to 5FU was associated with low TS levels and high TS inhibition, but that in the same patients the incorporation into RNA was not associated with response.[14]

This information should be used in the selection of novel combinations, such as oxaliplatin and irinotecan,[15,16] which should be aimed at increasing the TS inhibition in the tumor, since a higher incorporation into RNA would enhance toxic side effects.

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