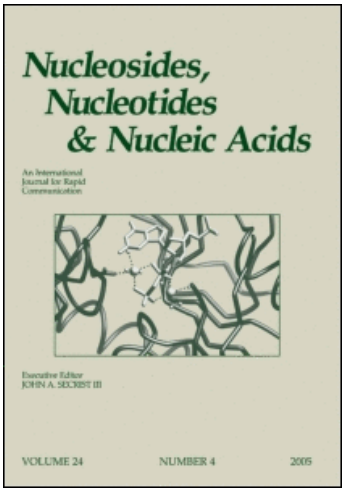

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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To cite this Article Giovannetti, E. , Backus, H. H. J. , Wouters, D. and Peters, G. J.(2008) 'Functional Inactivity and Mutations of p53 Differentially Affect Sensitivity to 5-Fluorouracil and Antifolate Inhibitors of Thymidylate Synthase (TS) by Altering TS Levels in Colorectal Cancer Cells', Nucleosides, Nucleotides and Nucleic Acids, 27: 6, 740 — 745
To link to this Article: DOI: 10.1080/15257770802145512
URL: http://dx.doi.org/10.1080/15257770802145512

Nucleosides, Nucleotides, and Nucleic Acids, 27:740–745, 2008

ISSN: 1525-7770 print / 1532-2335 online
DOI: 10.1080/15257770802145512

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FUNCTIONAL INACTIVITY AND MUTATIONS OF p53 DIFFERENTIALLY AFFECT SENSITIVITY TO 5-FLUOROURACIL AND ANTIFOLATE INHIBITORS OF THYMIDYLATE SYNTHASE (TS) BY ALTERING TS LEVELS IN COLORECTAL CANCER CELLS

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□ *The role of p53 in altering TS expression and chemosensitivity was studied in colorectal cancer cells with wildtype, mutated, or functionally inactive p53. Cytotoxicity of TS inhibitors was studied by MTT, while PCR, Western blot, and activity assays assessed whether p53 status influenced TS expression. Lovo-175X2 cells showed increased resistance to TS inhibitors and significantly greater than wildtype expression and activity of TS. In contrast, Lovo-273X17 and Lovo-li were more sensitive to TS inhibitors and had reduced TS expression, due either to reduced TS mRNA or altered regulation of TS activity. Thus, functional inactivity and mutations of p53 differentially affect TS, potentially influencing response to TS inhibitor-based treatment.*

Keywords p53; thymidylate synthase; thymidylate synthase inhibitors; colorectal cancer

INTRODUCTION

Fifty years after its introduction, 5-fluorouracil (5-FU) remains part of the standard treatment for colorectal cancer (CRC), with, however, limited efficacy as a single agent (response rate $< 20\%$).[1] The identification of markers to predict 5-FU response in CRC is warranted. The main cellular target of 5-FU is thymidylate synthase (TS), a key enzyme in the de novo synthesis of dTMP, an essential precursor for DNA replication.[2] Since high expression of TS is associated with poor responsiveness to 5-FU,[3] analysis of determinants of TS expression may allow the prediction of tumor response to chemotherapy.

The tumor suppressor gene p53, which is mutated in 50% of CRC, regulates the expression of several genes. Previous studies have shown that wildtype (wt) human p53 protein can inhibit transcription of the mouse TS promoter[4] and that a significant increase in TS expression and catalytic

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activity might be caused by the loss of the inhibitory effect of wt p53 on the activity of TS promoter in human CRC cells.[5] However, no clear survival benefit for wt p53 was observed in 5-FU treated CRC patients,[6] and the presence of p53 mutations failed to predict which patients would benefit from 5-FU-based adjuvant chemotherapy in large retrospective clinical trials.[7]

This study aimed to clarify the role of p53 in altering TS levels and sensitivity to TS inhibitors. Wildtype p53 colorectal cancer cells Lovo-92 and its mutated transfected variants Lovo-175X2 and Lovo-273X17, as well as Lovo-li cells (harboring a functionally inactive p53) were tested for chemosensitivity to 5-FU and several antifolate inhibitors of TS (pemetrexed, nolatrexed, raltitrexed and GW1843). Characterization of these cell types with regard to TS mRNA and protein expression and TS activity levels demonstrated that different mutations and functional activity of p53 can differentially alter sensitivity to TS inhibitors.

MATERIALS AND METHODS

5-FU was provided by Sigma Chemicals Co. (St. Louis, MO, USA), raltitrexed by Astra-Zeneca Pharmaceuticals (Macclesfield, UK), pemetrexed by Eli Lilly Inc. (Indianapolis, IN, USA), GW1843U89 by Glaxo/Wellcome Co. (Research Triangle Park, NC, USA) and nolatrexed by Zarix, Limited (King of Prussia, PA, USA). All other chemicals were of analytical grade and were obtained from commercial sources.

The human colon carcinoma cell lines Lovo-92 and their transfected variants were generously provided by Dr. Poupon and functional activity of Lovo-li was determined as described previously.[6]

Chemosensitivity, FdUMP binding and TS catalytic activity were measured as described previously,[8] TS mRNA expression was studied using quantitative PCR, as described before,[9] while Western blot analysis of TS and p53 were performed according to Backus et al.[8]

RESULTS

To study a potential effect of p53 mutations on the sensitivity to TS inhibitors, we performed growth inhibition experiments in Lovo-92 colorectal cancer cells and their transfected variants. No significant differences in doubling times were observed between the Lovo variants (Table 1). For the empty vector plasmid control Lovo-B2 and the parental cell line Lovo-92 no significant differences ($P > 0.05$) were observed in sensitivity for all drugs, although Lovo-B2 tended to be more sensitive to all the antifolates. Transfection with mt p53 induced resistance in Lovo-175X2 to 5-FU and all antifolates tested except GW1843 ($P < 0.05$) compared with the wt p53 parental cell line Lovo-92. Resistance in Lovo-175X2 varied from 2-fold for 5-FU to

TABLE 1 IC_{50} values of TS inhibitors and doubling times in Lovo cell lines

	P53 status	Doubling time (hours)	5-FU (μM)	Nolatrexed (μM)	Raltitrexed (nM)	Pemetrexed (nM)	GW1843 (nM)
Lovo 92	wt p53; parental	34.5 ± 3.4	1.73 ± 0.15	5.15 ± 0.85	30.7 ± 0.7	417.0 ± 83.0	5.5 ±1.5
Lovo B2	Plasmid control	44.7 ± 4.0	1.87 ± 0.58	4.57 ± 0.72	21.3 ± 7.2	325.0 ± 104.1	2.4 ± 0.8
Lovo 175X2	Transfected with mt p53	46.5 ± 5.5	3.25 ± 0.25*	19.00 ± 1.00*	100.0 ± 1.0*	3912.0 ± 138.9*	5.3 ± 0.3
Lovo 273X17	Transfected with mt p53	38.3 ± 3.5	0.30 ± 0.08*	4.75 ± 0.20	23.3 ± 4.4	240.0 ± 49.0	3.0 ± 1.0
Lovo li	Functional inactive p53	41.7 ± 4.2	2.10 ± 0.40	2.10 ± 0.10*	5.7 ± 0.7*	72.5 ± 12.5*	0.8 ± 0.3

IC_{50} values are given as mean values (in nM or μM) ± standard error of the mean (SEM) of at least three experiments. Significant differences ($P < 0.05$) between parental and transfected cells are indicated with*. Sensitivity data have been partially reported previously.[5]

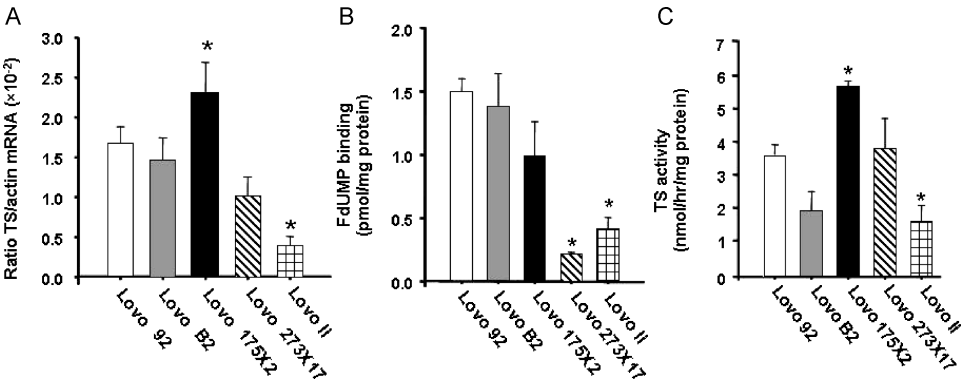

FIGURE 1 TS levels in Lovo cells. (A) TS mRNA levels were normalized to the simultaneously determined β-actin mRNA expression as described in the Material and Methods section. (B) FdUMP binding (pmol/mg protein) and (C) TS activity (nmol/hr/mg protein) were measured in cell extracts. Results are given as mean values ± SEM of at least three experiments. Significant differences ($P < 0.05$) between parental and transfected lines are indicated with*.

10-fold for pemetrexed. In contrast, the inactive status of p53 in Lovo-li reduced IC_{50} values of nolatrexed, raltitrexed, pemetrexed and GW1843 by 3- to 15-fold. In line with this, the other mt p53 transfected Lovo cell line, 273X17, with a mutation at the position 273 (Arg → His), was 6-fold more sensitive to 5-FU compared with Lovo-92 ($P < 0.05$).

PCR analysis of TS mRNA expression showed no significant differences among Lovo-92, Lovo-B2 and Lovo-273X17, while TS expression was 1.5-fold higher in Lovo-175X2 in comparison to Lovo-92 cells. In contrast, Lovo-li cells were characterized by a significantly reduced expression of TS, compared to the parental cells (Figure 1A).

The transfection of wt and mt p53 was checked by analysis of p53 protein expression using Western blot analysis (Figure 2). As expected, p53 expres-

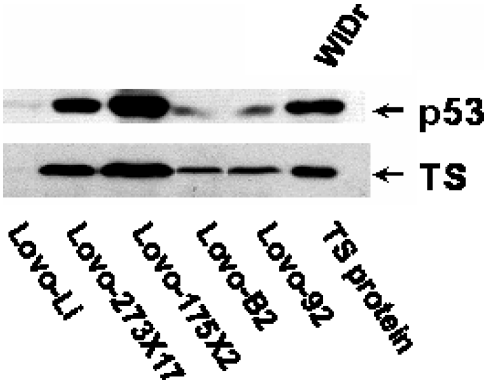

FIGURE 2 p53 and TS protein expression in Lovo cells. Representative blot of at least three independent Western blotting analyses performed as described in the Material and Methods. WiDr cells (25 μg total protein) and TS control protein (1 ng) were used as positive controls for the expression of p53 and TS, respectively.

sion was low in wt p53 cell lines Lovo-92 and Lovo-B2, while up-regulation was found in the mt p53 transfectants Lovo-175X2 and Lovo-273X17. Little p53 expression was detected in the p53 inactive cell line Lovo-li. The status of p53 mRNA expression was associated with changes in the protein expression of TS. Low TS protein levels were detected in Lovo-92 and Lovo-B2 cells, whereas an increase was found in Lovo-175X2 and 273X17, more pronounced in Lovo-175X2. Finally, no TS protein was detectable in Lovo li.

Changes in TS protein expression were accompanied by differences in TS activity levels (Figures 1B and 1C). Lovo-li and Lovo-273X17 had significantly less FdUMP binding sites compared with Lovo-92, Lovo-B2, and Lovo-175X2 cells. In addition, the catalytic activity of TS was about 2-fold lower in Lovo-li compared with the parent cell line, while TS activity was about 1.5 higher in Lovo-175X2 ($P < 0.05$).

DISCUSSION

The present study demonstrates that changes in the status of p53 due to mutations or inactivity can alter sensitivity to TS inhibitors such as 5-FU and antifolates. Sensitive p53 inactive Lovo cells had low levels of TS whereas high TS levels were found in resistant mt p53 cells. Mutations in the tumor suppressor gene p53 have been correlated with chemoresistance,[10] and, in a panel of 14 colon cancer cell lines, we observed that cells with a p53 mutation were more resistant to 5-FU and raltitrexed.[3]

Transfection of mt p53 into Lovo-175X2 cells increased TS mRNA and protein expression and activity, which were associated with a decreased sensitivity to 5-FU and antifolates compared to the wt parental cell line Lovo-92. The increase in TS levels might be caused by the loss of the inhibitory effect of wt p53 on the promoter activity of TS.[5]

In contrast to the case in mt p53 Lovo-175X2 cells, functionally inactive p53 in Lovo li cells was associated with increased sensitivity to TS inhibitors which as well as with decrease TS mRNA, protein, and activity levels.

Similar but less pronounced results were obtained after transfection of mt p53 in Lovo 273X17. The differences among levels of TS mRNA, TS protein and TS activity in Lovo 175X2 and Lovo 273X17 may be related to the different mutation affecting p53 in these transfected cell lines cells (i.e., mutation at position 175 (Arg $\rightarrow$ His) in Lovo 175X2 and at position 273 (Arg $\rightarrow$ His) in Lovo 273X17 cells). These results are in line with those found by Blandino et al.,[11] who demonstrated that different mutations in p53 can induce different effects on the sensitivity of cultured cells to chemotherapy.

These data might also explain why immunohistochemistry of p53 does not reliably predict survival benefit in colorectal cancer patients treated with 5-FU based chemotherapy,[6] although the lack of relationship with clinical outcome may also be related to the discrepancy between p53 immunostaining and mutation analysis.[12]

In conclusion, changes in the status of p53 due to mutations or functional inactivity change the sensitivity to TS inhibitors by altering the expression and activity of TS. The increase and decrease in sensitivity to TS inhibitors in vitro might explain why no clear correlation was found in clinical studies between mutations in p53 and clinical outcome. Analysis of the status of p53 (e.g., wt or mt and functionally active or not) could, however, be useful to predict clinical outcome after chemotherapy with TS inhibitors.

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