symposium article

Annals of Oncology 17 (Supplement 5): v25–v28, 2006
doi:10.1093/annonc/mdj945

Topoisomerase I, IIα and IIβ mRNA expression in peripheral blood mononuclear cells of patients with solid tumor: preliminary results

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Background: The pyrimidine antimetabolite Gemcitabine (G) (2′,2′-difluorodeoxycytidine) is used against several malignancies G exerts its antitumour effect mainly by incorporation of its triphosphate metabolite (dFdCTP) into DNA. Subsequently, DNA polymerase adds one additional deoxynucleotide and DNA synthesis is interrupted. The nuclear enzymes topoisomerase I and II (TPs) are critical for DNA function and cell survival; they control, maintain and modify DNA topology during both replication and translation of genetic materials. These enzymes induce cuts in one or both strands of DNA, allowing strands to pass through the nick and then rejoining the nicked strand of DNA. Anti-topoisomerase (TPs-inhibitors) drugs exist and are largely used in chemotherapy, however, most often blindly of the cancer TPs status.

Aim: To understand the best association between G and TPs-inhibitors, we studied: (a) Topoisomerases I, IIα and IIβ mRNA expression in Peripheral Mononuclear Blood Cells (PBMCs) of patients with solid tumor, after 1, 2, 3, 4, 5, 6 h after treatment with Gemcitabine (G); b) *in vivo* expression of TPs genes after administration of Gemcitabine (a topoisomerases up-regulating drug) combined with the TPs inhibitors drugs (TID) Topotecan (T) and Etoposide (E), added to the culture beneath 1 h after TPD treatment. TPs mRNA expression was measured by quantitative real-time RT-PCR in PBMCs.

Results: The administration of 1-h infused G is followed by a fast rise of TPs expression ($P > 0.0001$ Student's t test, paired data, each patient control of himself); TPs inhibitors, sequentially given after G, highly reduced the TPs rising ($P > 0.0001$).

Conclusions: G induces a TPs increase. A rationale might be available for combination chemotherapy (G plus TPs inhibitors). The study is ongoing to enrol further patients.

Key words: gemcitabine, topoisomerases enzymes, mRNA expression, real-time RT- PCR, solid tumor, topoisomerases-inhibitors

introduction

Topoisomerases (TPs) are enzymes of the DNA gyrase family, and participate in every metabolic function of DNA, including replication, transcription and other essential roles of cellular processes [1–4]. They belong to prokaryotic as well as to eukaryotic cells [5–6], and they are supposed to have been expressed in the ancient primordial cell [7]. Genes coding for the topoisomerase enzymes are highly conserved across species.

Two types of topoisomerases are known: type I, that modifies DNA linking number by passing a single nucleic acid strand through a transient break, and type II (α and β) that catalyses a similar process using ATP, producing a double stranded DNA break [8–10] (Table 1). TPs reduce DNA twisting and supercoiling, that occur in selected regions of DNA. They possess two characteristics, as it has been finely demonstrated by Stewart and Ratain: to cleave and reseal the phosphodiester backbone of DNA, and to form a covalent enzyme-DNA linkage which allows the passage of another single or double-strand DNA through the nicked DNA [9]. Thus, TPs enable the DNA to remain tightly packed and yet still accessible for the process that is necessary for proper cellular functions [10–12].

Anti-topoisomerase drugs have been introduced in the treatment of patients with a wide range of malignancies with appreciable effectiveness in selected cancers [11–12]. These drugs include the type 1 anti-topoisomerases (anti-TP1), as the camptothecin analogues, introduced in the anticancer therapy only from few years, and anti-TP2, as the classic anthracyclines and epipodophyllotoxines. Typically, these drugs do not inhibit the free enzyme but stabilize the covalently-bound topoisomerase–DNA complex [13].

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Gemcitabine (dFdC, 2′,2′-difluoro-2′-deoxycytidine) is a deoxycytidine analogue with a broad spectrum of anticancer activity against several solid tumours in preclinical models [14], and it is now an established effective agent in the treatment of different type of cancer [15]. G is assumed to exert its antitumour effect mainly by incorporation of its triphosphate metabolite (dFdCTP) into DNA, after which DNA polymerase adds one additional deoxynucleotide and DNA synthesis is interrupted. The rate-limiting step in the activation of the drug is catalysed by deoxycytidine kinase (dCK), which is a limiting factor for the cytotoxic activity of gemcitabine. Furthermore, the diphosphate metabolite (dFdCDP) inhibits ribonucleotide reductase (RR), an enzyme that converts ribonucleotides to deoxyribonucleotides, required for DNA polymerisation and repair. Ribonucleotide reductase consists of dimerised large and small subunits 1 and 2 (RRM1 and RRM2), whose pairing is essential for scheduled DNA synthesis to occur. The interaction between G and RR has not been well characterised; however, data support the hypothesis that the RRM1 subunit is the most important intracellular target of dFdCDP [16], although resistance to G was observed both in RRM1- and RRM2-overexpressing cells [17, 18]. Thus, G resistance may be dependent on decreased expression of dCK, or overexpression of RR.

Our group (Padua Pharmacology Oncology Group, PPOG) is studying the behaviour of TP-I, IIα, IIβ, both *in vitro* and *in vivo* (present clinical studies). In this research the levels of TPs mRNA expression were determined in peripheral blood mononuclear cells (PBMC) by means of quantitative real-time RT-PCR performed using a LightCycler rapid thermal cycler system (Roche Diagnostics Ltd., Lewes, UK) and 7700 sequence detector system (Applied Biosystem) [19].

patients and methods

Patients receiving i.v. G 1000 mg/m^2 over 1 h were 45; 20 F, 25 M; aged 40 to 83 (mean 66.3; median 66), with different malignancy. Blood sampling was pre-G (basal), at the G administration end, and hourly thereafter (up to the 6th hour). Cancer types were: NSCLC (14 patients), ovarian (9), breast (7), pancreatic (7), kidney clear cell (4), hepatic (2) and pleural mesothelioma (2). All patients gave their written informed consent.

Table 1. Primers and probes used in 20 μl reaction mixture

Name	Sequence
TP1 Forward primer	5′-TGACAGCCCCGGATGAGA-3′
Reverse primer	5′-TGCCAATCGAGCTGTTGCA-3′
Probe	5′-CATCCCAGCGAAGATCCTTTCTTATAACCG-3′
TP2α Forward primer	5′-TTGAAGACGCTTCGTTATGGG-3′
Reverse primer	5′-CCATCACAACTGGCCCTCTC-3′
Probe	5′-ACAGATCAGGACCAAGATGGTTCCCACAT-3′
TP2β Forward primer	5′-GCGATTATAACCCTGGCAGGT-3′
Reverse primer	5′-CAGACTTCCCTACTGAGCCACC-3′
Probe	5′-AACATCCAAACAACAAGCAAGAAACCGAA-3′

isolation of total RNA and cDNA synthesis

Total RNA was prepared by Omnizol® reagent (Euroclone, Ltd. UK) according to the manufacturer's instructions. Briefly, Omnizol® reagent was used to lyse the PBMCs samples, followed by chloroform extraction, and RNA precipitation with isopropyl alcohol. The isolated tumor RNA was resolved in sterile water containing 0.2% (v/v) diethylpyrocarbonate (DEPC). Three micrograms of RNA were transcribed into cDNA by *MuLv* reverse transcriptase (Promega) with random oligonucleotide primers in a final volume of 60 μl.

quantitative RT-PCR

The expression of RPLPo gene was used as internal control, and was quantified to compensate for differences in the RNA quality of RT efficacy in our dilution series and samples. Primers and TaqMan probes for top I, IIα and IIβ were designed by using Primer Express™ (Applied Biosystems, Sweden) (Table 1).

6-Carboxyfluorescein (FAM) was used as reporter and 6-carboxy-tetramethyl-rhodamine (TAMRA) as the quencher dye.

The PCR amplification was performed in a 96-well tray with optical caps (Applied Biosystems, Sweden) with a 25 μl final reaction mixture. The fluorescence signal was monitored on-line using the laser detector of the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Sweden).

evaluation of real-time RT-PCR results

The initial template concentration was derived from the cycle number at which the fluorescent signal crossed a threshold in the exponential phase of the polymerase chain reaction (CT-value). As there was a very large variation of mRNA level in patient samples, we have adjusted the template concentrations so that the CT-values from patient samples in all cases were within the range of the standard curve. The target CT-values were then compared with the no template controls and translated into target amplification value. The results of Top I, IIα or IIβ relative mRNA levels in PBMCs were expressed as their amplification value divided by amplification value of internal control RPLPO.

results

TPs expression in 45 patients treated with G

G (1000 mg/m^2, per 60 min infusion, days 1, 8, and 15) was intravenously given to all 45 patients. Blood sampling were done basally (0) and then after 1, 2, 3, 4, and 5 hours after chemotherapy start. Results of genetic expression, after G infusion, are reported in Figures 1–3. The increase of TP-I and

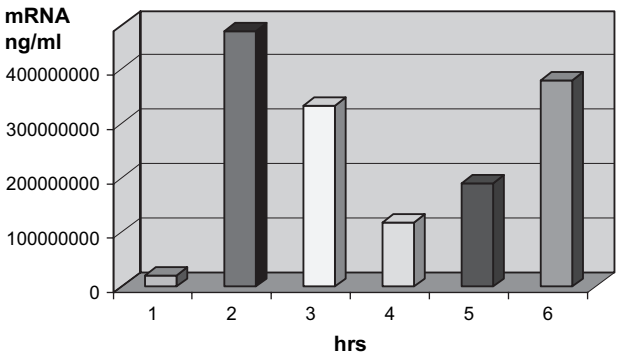

Figure 1. TP-I expression in PBMC of 45 pts. Treated with G. Blood sampling at 0 (1), 2 (2), 3 (3), 4 (4), 5 (5), 6 (6) hours from therapy starting.

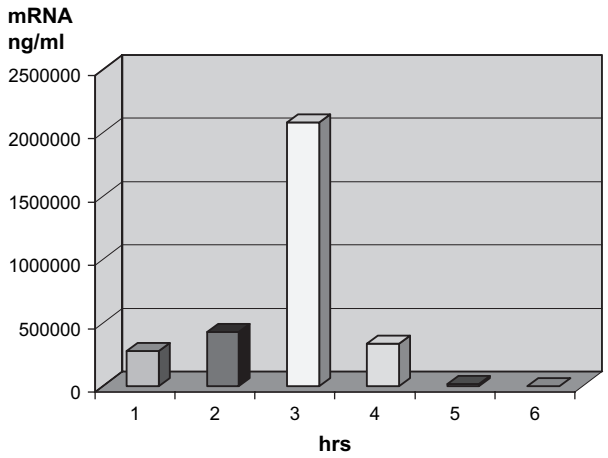

Figure 2. TP-IIα expression in PBMC of 45 pts. Treated with G. Blood sampling at 0 (1), 2 (2), 3 (3), 4 (4), 5 (5) hours from therapy starting.

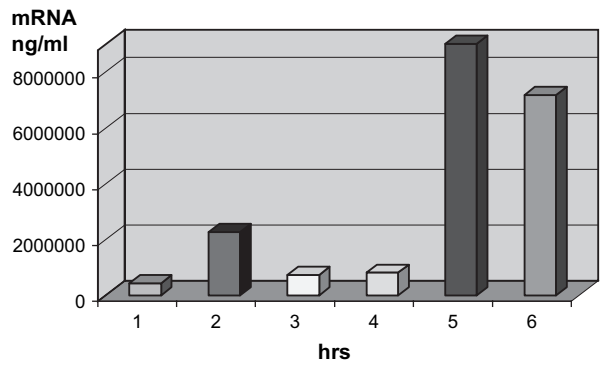

Figure 3. TP-IIβ expression in PBMC of 45 pts. Treated with G. Blood sampling at 0 (1), 2 (2), 3 (3), 4 (4), 5 (5), 6 (6) hrs from therapy starting.

TP-IIβ has a biphasic behaviour, with a first pick at 2nd hour and a second at 5th hour from the chemotherapy starting. Indeed, the increase of TP-IIα has its peak at 2nd hour (Figures 1–3; note the different y scaling in the three graphs). Also, the percentages of increase of the three enzymes after G infusion are different; a greater increase is observed in TP-I, with a median value of 1384%, followed by TP-IIβ, which medium percentage increase of 865%, and then by TP-IIα (119%).

TPs expression in pts treated with G immediately followed by anti-TPs drugs

As in our patients treated with G the TPs levels increased consistently, we decided to study how the expression of TPs genes changes after administration of G (topoisomerases promoting drug) combined with the TPs inhibitors drugs (TID) Topotecan (T) and Etoposide (E). So, 21 days after the first G administration, 23 of the above quoted pts received G sequentially followed by Topotecan and Etoposide (TPs inhibitors) over 1 h infusion each. Such a sequence of the anti-topoisomerases drugs was based on the *in vitro* study by Whitacre CM et al. [20]. Blood sampling was identically repeated, as done before.

Results show an important decrease of the TPs expression after anti-TPs administration (Figures 4–6), with a median

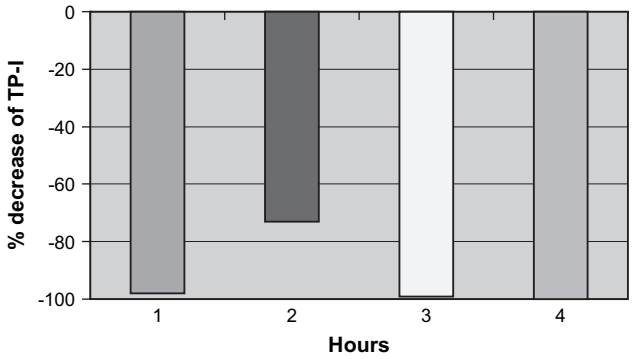

Figure 4. Decrease (%) of the expression of TP-I in PBMC of 23 pts treated with intravenous G, immediately followed by Topotecan and Etoposide. Blood sampling at 0 (1), 2 (2), 3 (3), 4 (4) hrs after chemotherapy administration.

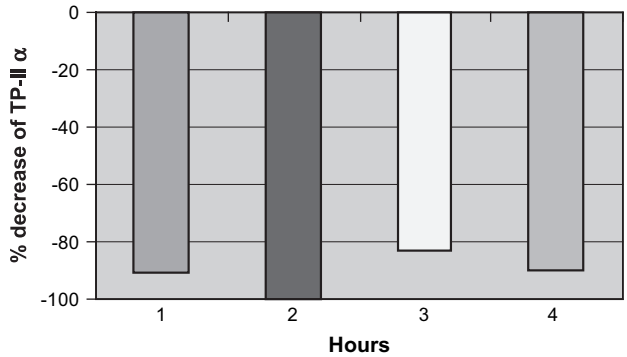

Figure 5. Decrease (%) of the expression of TP-IIα in PBMC of 23 pts treated with intravenous G, immediately followed by Topotecan and Etoposide. Blood sampling at 0 (1), 2 (2), 3 (3), 4 (4) hours after chemotherapy administration.

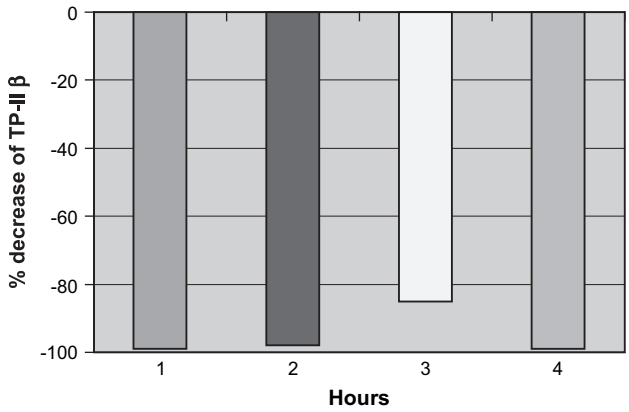

Figure 6. Decrease (%) of the expression of TP-IIβ in PBMC of 23 pts treated with intravenous G, immediately followed by Topotecan and Etoposide. Blood sampling at 0 (1), 2 (2), 3 (3), 4 (4) hrs after chemotherapy administration.

percentage decrease of 93% (TP-I), 91% (TP-IIα) and 95% (TP-IIβ).This TPs expression decrease was compared with the TPs expression observed after G alone, and in each instance the statistical difference was highly significant (P <0.0001).

discussion

We evaluated the TPs expression in the peripheral blood mononuclear cells (PBMCs) of cancerous patients treated with Gemcitabine. Aim of this study was to detect the behaviour of TPs zenith. This could help to choice the most useful time schedule which G and anti-TPs drugs could be administered.

Present results show that the administration of G (1-h infusion) is followed by a steepy and statistically significant rise of TPs expression, within the order of 1384%, (TP-I), 865% (TP-IIβ), and 119% (TP-IIα), with a zenith within the third hour. The administration of the TPs- inhibitors drugs, given immediately after the Gemcitabine infusion, reduced the TPs rising (P <0.0001 Student's t test, paired data, each patients control of himself).

Present study is ongoing to enrol further patients; if the hypothesis that G induces a cancer host defence at the level of TPs increase will be confirmed, a rationale could be available for combination therapy of G plus TPs inhibitors.

In vitro studies on cancer cells (see the Richter's results, in this journal number) supporting the clinical pharmacology.

disclosures

Professor Cartei has indicated no financial relationships with companies whose products are mentioned in this article.

acknowledgements

Present work is a part of the study program, and partly supported by AOI (Associazione Oncologica Italiana), and by Regione Veneto and IOV-IRCCS.

references

1. Liu LF. DNA topoisomerases–enzymes that catalyse the breaking and rejoining of DNA. CRC Crit Rev Biochem 1983; 15: 1–24.
2. Wang JC. DNA topoisomerases. Annu Rev Biochem 1996; 65: 635–692.
3. Gupta M, Fujimori A, Pommier Y. Eukaryotic DNA topoisomerases I. Biochem Biophys Acta 1995; 1262: 1–14.
4. Strausfeld U, Richter A. Simultaneous purification of DNA topoisomerase I and II from eukaryotic cells. Prep Biochem 1989; 19: 37–48.
5. Iyer LM, Koonin EV, Aravind L. Evolutionary connection between the catalytic subunits of DNA-dependent RNA polymerases and eukaryotic RNA-dependent RNA polymerases and the origin of RNA polymerases. BMC Struct Biol 2003; 3: 1.
6. Perry K, Mondragon A. Structure of a complex between *E. coli* DNA topoisomerase I and single-stranded DNA. Structure (Camb) 2003; 11: 1349–58.
7. Grabowski B, Kelman Z. Archeal DNA replication: eukaryal proteins in a bacterial context. Annu Rev Microbiol 2003; 57: 487–516.
8. Zechiedrich EL, Christiansen K, Andersen AH et al. Double-stranded DNA cleavage/religation reaction of eukaryotic topoisomerase II: evidence for a nicked DNA intermediate. Biochemistry 1989; 28: 6229–6236.
9. Gatto B, Richter S, Moro S et al. The topoisomerase II poison clerocidin alkylates non-paired guanines of DNA: implications for irreversible stimulation of DNA cleavage. Nucleic Acids Res 2001; 29: 4224–4230.
10. Stewart L, Champoux JJ. Assaying DNA topoisomerase I relaxation activity. Methods Mol Biol 2001; 95: 1–11.
11. Turner PK, Iacono LC, Stewart CF. Topoisomerase I interactive agents. Cancer Chemother Biol Response Modif 2003; 21: 69–101.
12. Staker BL, Hjerrild K, Feese MD et al. The mechanism of topoisomerase I poisoning by a camptothecin analog. Proc Natl Acad Sci USA 2002; 99: 15387–15392.
13. Rubin EH. DNA topoisomerase expression in tumors-a novel target for chemioterapy. Human Pathol 2000; 31: 631–632.
14. van Moorsel CJ, Bergman AM, Veerman G et al. Differential effects of gemcitabine on ribonucleotide pools of twenty-one solid tumour and leukaemia cell lines. Biochim Biophys Acta 2000; 1474: 5–12.
15. Noble S, Goa KL. Gemcitabine. A review of its pharmacology and clinical potential in non-small cell lung cancer and pancreatic cancer. Drugs 1997; 54: 447–472.
16. van der Donk WA, Yu G, Perez L et al. Detection of a new substrate-derived radical during inactivation of ribonucleotide reductase from *Escherichia coli* by gemcitabine 5′-diphosphate. Biochemistry 1998; 37: 6419–2646.
17. Goan YG, Zhou B, Hu E et al. Overexpression of ribonucleotide reductase as a mechanism of resistance to 2,2-difluorodeoxycytidine in the human KB cancer cell line. Cancer Res 1999; 59: 4204–4207.
18. Davidson JD, Ma L, Flagella M et al. An increase in the expression of ribonucleotide reductase large subunit 1 is associated with gemcitabine resistance in non-small cell lung cancer cell lines. Cancer Res 2004; 64: 3761–3766.
19. Zhou R, Frostvik Stolt M, Kronenwett U et al. Real-time RT-PCR for the determination of topoisomerase II mRNA level in leukaemic cells. Leukemia Res 2002; 26: 487–494.
20. Whitacre CM, Zborowska E, Gordon NH et al. Topotecan increases topoisomerase IIalpha levels and sensitivity to treatment with etoposide in schedule-dependent process. Cancer Res 1997; 57: 1425–1428.