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Role of tyrosyl-DNA phosphodiesterase 1 and inter-players in regulation of tumor cell sensitivity to topoisomerase I inhibition

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ABSTRACT

Tyrosyl-DNA phosphodiesterase 1 (TDP1) plays a unique function as it catalyzes the repair of topoisomerase I-mediated DNA damage. Thus, ovarian carcinoma cell lines exhibiting increased TDP1 levels and resistance to the topoisomerase I poisons campthotecins were used to clarify the role of this enzyme. The camptothecin gimatecan was employed as a tool to inhibit topoisomerase I because it produces a persistent damage. The resistant sublines displayed an increased capability to repair drug-induced single-strand breaks and a reduced amount of drug-induced double-strand breaks, which was enhanced following TDP1 silencing. In loss of function studies using U2-OS cells, we found that TDP1 knockdown did not produce a change in sensitivity to camptothecin, whereas co-silencing of other pathways cooperating with TDP1 in cell response to topoisomerase I poisons indicated that XRCC1 and BRCA1 were major regulators of sensitivity. No change in cellular sensitivity was observed when TDP1 was silenced concomitantly to RAD17, which participates in the stabilization of collapsed replication forks. The expression of dominant-negative PARP1 in cells with reduced expression of TDP1 due to a constitutively expressed TDP1 targeting microRNA did not modulate cell sensitivity to camptothecin. Mild resistance to gimatecan was observed in cells over-expressing TDP1, a feature associated with decreased levels of drug-induced single-strand breaks. In conclusion, since TDP1 alone can account for mild levels of camptothecin resistance, repair of topoisomerase I-mediated DNA damage likely occurs through redundant pathways mainly implicating BRCA1 and XRCC1, but not RAD17 and PARP1. These findings may be relevant to define novel therapeutic strategies.

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1. Introduction

Topoisomerase I (Topo I) is a nuclear enzyme that plays an essential role in DNA replication, transcription, recombination and repair [1,2]. The enzyme catalyzes changes in the topology of DNA by transiently breaking one strand of DNA with covalent attachment to the 3′ terminus of the nicked DNA leading to the formation of a Topo I covalent complex (i.e., cleavable complex) through a tyrosine hydroxyl group of the enzyme. Topo I is the cellular target of camptothecins (CPTs), which act specifically at the level of the DNA–Topo I complex and, through stabilization of the complex, they stimulate DNA cleavage [1,2]. CPTs are used in the treatment of a variety of human tumors, including ovarian carcinoma [3,4].

In spite of the therapeutic potential of Topo I inhibitors, development of resistance is a major limitation to the success of antitumor therapy with CPTs [5,6]. Since the critical event in the cytotoxic effect of CPTs is the formation of the ternary DNA–Topo I–drug complex, cellular resistance to CPTs can occur by mechanisms reducing the formation of the cleavable complex. Alterations leading to reduced drug–target interaction such as decreased drug accumulation resulting from over-expression of ATP binding cassette transporters [7–11]; mutations of the Topo I

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gene [6,12–14]; increased Topo I degradation through ubiquitination or sumoylation have been described in cells selected *in vitro* for resistance to CPTs [15,16]. CPT-resistant cells have been shown to exhibit increased expression of anti-apoptotic proteins [17]. Increased repair of CPT-induced lesions has been reported in CPT-resistant cells. For example, enhanced nucleotide excision repair activity was found in breast cancer cells selected for resistance to CPT [18]; CPT-resistant cell lines exhibit increased XRCC1 levels and transfection of XRCC1 confers CPT resistance [19]. Overexpression of tyrosyl-DNA phosphodiesterase 1 (TDP1) in human cells through transfection has also been associated with decreased damage following exposure to high concentration of CPT [20]. A fraction of the TDP1 enzyme localizes to the mitochondria and acts in repair of mitochondrial DNA [21]. Recently, a human 5′-tyrosyl DNA phosphodiesterase (TTRAP/TDP2) that can cleave 5′-phosphotyrosyl bonds has been implicated in cellular resistance to topoisomerase poisons [22,23].

The 7-substituted lipophilic camptothecin derivative gimatecan – currently undergoing Phase II clinical evaluation – appears a promising drug for the treatment of solid tumors [4]. Cellular studies documented a high growth-inhibitory potency in several human tumor cell lines and the capability to overcome multidrug resistance mediated by different transporters [24–26]. Preclinical evaluation of the antitumor efficacy in human tumor xenografts indicated potent antitumor activity and improved pharmacological profile as compared to topotecan (TPT) [25]. Moreover, among CPTs, gimatecan is a good experimental tool because it produces Topo I cleavable complexes more stable than those formed by other CPTs [24]. Thus, in the present study, we examined the role of TDP1, an enzyme implicated in the repair of strand breaks arising from abortive Topo I complexes, and its putative inter-players in modulating sensitivity to CPTs taking advantage of ovarian carcinoma cell lines resistant to gimatecan generated in our laboratory, exhibiting enhanced TDP1 levels.

2. Materials and methods

2.1. Drugs

Gimatecan (7-terbutoxyiminomethylcamptothecin) [24], SN38, NSC 120686 and NSC 128609 (Sigma–Aldrich Discovery CPR, Milan, Italy) were dissolved in DMSO and diluted in water; TPT and doxorubicin were dissolved in water; etoposide (Vepesid, Bristol-Myers Squibb, Roma, Italy) was dissolved in ethanol and diluted in culture medium.

2.2. Cell lines and growth conditions

The human ovarian carcinoma IGROV-1 cell line [27], the gimatecan-resistant IGROV-1CPT/L and IGROV-1CPT/H sublines, cultured in RPMI-1640 medium, and the human osteosarcoma U2-OS cell line (ATCC), grown in Mc Coy's 5A medium were used. Medium was supplemented with 10% foetal calf serum. Resistant sublines were generated by chronic exposure to increasing gimatecan concentrations starting from 0.011 up to 0.2 μM. Such sublines were designated as IGROV-1CPT/L and IGROV-1CPT/H according to their lower (L) and higher (H) degree of resistance obtained in comparison to parental cells. The phenotype of the studied resistant variants was stable at least for 6 months when cells were grown in the absence of drug. Resistant cells were routinely cultured in the absence of gimatecan. The growth characteristics of sensitive and resistant cells were similar. Polyclonal populations of cells stably transfected with the TDP1 containing vector (U2-OS/TDP1) or empty vector (U2-OS/e) were grown in the presence of 400 μg/ml G418 (Calbiochem Inalco, Milan, Italy). U2-OS cells stably expressing miRNAs were cultured in the presence of blasticidin (2.5 μg/ml, Invitrogen, San Giuliano Milanese, Italy).

2.2.1. Cell sensitivity to drugs

The cell sensitivity to antitumor agents was measured by using the growth-inhibition assay based on cell counting or the sulforhodamine B assay (SRB) [28]. For the former assay, cells were seeded in duplicates into 6-well plates and exposed to drug 24 h later. After 1 h of drug incubation, the medium was replaced with fresh medium. Cells were harvested 72 h later for counting with a cell counter. For the SRB assay, cells were seeded in 96-well plates and, 24 h later, they were treated with drugs for 48 or 72 h. IC_{50} is defined as the drug concentration producing 50% decrease of cell growth/absorbance. For each assay, at least three independent experiments were performed.

2.3. Topo I activity in nuclear extracts

Cells (5×10^6) were harvested after trypsinization, washed with PBS, and resuspended in 5 ml of nuclear buffer [1 mM KH_2PO_4 (pH 6.4), 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 10 mM 2-mercaptoethanol, 10% (vol/vol) glycerol]. After addition of 45 ml of nuclear buffer containing 0.35% (vol/vol) Triton X-100 and 0.5 mM PMSF, the cell suspension was kept on ice for 30 min. The nuclei were collected by centrifugation at $1000 \times g$ for 10 min, washed once with Triton X-100-free nuclear buffer and then incubated for 1 h at 4 °C in lysis buffer [5 mM KH_2PO_4 (pH 7.0), 100 mM NaCl, 0.5 mM EGTA, 10 mM 2-mercaptoetanol, 10% (vol/vol) glycerol, 10 mM $NaHSO_3$] containing 0.35 M NaCl in gentle rotation. The lysate was centrifuged at $12{,}000 \times g$ for 15 min and the protein concentration was determined using the BioRad Protein Concentration Assay (Bio-Rad Laboratories Srl, Segrate, Italy). Equal amounts of the nuclear protein extract for each cell line were immediately assayed for Topo I activity. The activity of Topo I was determined by measuring the relaxation of supercoiled pBR322 DNA (Invitrogen). The reaction mixture (final volume 20 μl) containing 20 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mg/ml BSA, 150 mM NaCl, and 250 ng of pBR322, and different dilutions of nuclear extract was incubated at 37 °C for 30 min. The reaction was stopped by adding 1% SDS, 0.3 mg/ml proteinase K and incubating for 45 min at 42 °C. The samples were loaded on 1% agarose gel in TBE buffer (0.089 M Tris base, 0.089 M boric acid, and 0.002 M EDTA) and run over night at 40 V. After staining with 0.5 mg/ml ethidium bromide, the gels were photographed under UV light.

2.4. DNA sequence analysis of TOP1 and TDP1

The DNA sequence of TOP1 was analysed by RT-PCR. Total cellular RNA was isolated by using a commercially available kit (Talent, Trieste, Italy). Two micrograms of RNA was reverse transcribed into cDNA with the use of oligo-(dt) primers and Moloney murine leukaemia virus reverse transcriptase (Invitrogen). The entire TOP1 transcripts were amplified in two parts using 2 pairs of primers. The first pair of primes (5′-atgagtggggaccacctccacaa-3′/5′-ttcattagtcatttcctttctccagt-3′) gave a fragment of 867 bp, the second pair (5′-aatgctcgaccatgaatatactacc-3′/5′-taaaactcatagtcttcatcagcc-3′) amplified a DNA fragment of 1512 bp. The TDP1 transcripts were amplified using appropriate primers (5′-atgtctcaggaaggcgattatgggagg-3′/5′-tcaggagggcacccacatgttccc-3′). PCR conditions were as follows: 95 °C, 9 min for 1 cycle; 95 °C, 1 min, 52 °C for TOP1/62 °C for TDP1, 1 min, 72 °C, 1 min for 30 cycles followed by 10 min extension at 72 °C. The amplification products were purified on 1% agarose gel. Sequence analysis was performed by using the indicated primers and others

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