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Inside the biochemical pathways of thymidylate synthase perturbed by anticancer drugs: Novel strategies to overcome cancer chemoresistance

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ABSTRACT

Our current understanding of the mechanisms of action of antitumor agents and the precise mechanisms underlying drug resistance is that these two processes are directly linked. Moreover, it is often possible to delineate chemoresistance mechanisms based on the specific mechanism of action of a given anticancer drug. A more holistic approach to the chemoresistance problem suggests that entire metabolic pathways, rather than single enzyme targets may better explain and educate us about the complexity of the cellular responses upon cytotoxic drug administration. Drugs, which target thymidylate synthase and folate-dependent enzymes, represent an important therapeutic arm in the treatment of various human malignancies. However, prolonged patient treatment often provokes drug resistance phenomena that render the chemotherapeutic treatment highly ineffective. Hence, strategies to overcome drug resistance are primarily designed to achieve either enhanced intracellular drug accumulation, to avoid the upregulation of folate-dependent enzymes, and to circumvent the impairment of DNA repair enzymes which are also responsible for cross-resistance to various anticancer drugs. The current clinical practice based on drug combination therapeutic regimens represents the most effective approach to counteract drug resistance. In the current paper, we review the molecular aspects of the activity of TS-targeting drugs and describe how such mechanisms are related to the emergence of clinical drug resistance. We also discuss the current possibilities to overcome drug resistance by using a molecular mechanistic approach based on medicinal chemistry methods focusing on rational structural modifications of novel antitumor agents. This paper also focuses on the importance of the modulation of metabolic pathways upon drug administration, their analysis and the assessment of their putative roles in the networks involved using a meta-analysis approach. The present review describes the main pathways that are modulated by TS-targeting anticancer drugs starting from the description of the normal functioning of the folate metabolic pathway, through the protein modulation occurring upon drug delivery to cultured tumor cells as well as cancer patients, finally describing how the pathways are modulated by drug resistance development. The data collected are then analyzed using network/netwire connecting methods in order to provide a wider view of the pathways involved and of the importance of such information in identifying additional proteins that could serve as novel druggable targets for efficacious cancer therapy.

1. Introduction

Thymidylate synthase (TS) (E.C. 2.1.1.45) is a highly conserved enzyme, essential for cell survival due to its central role in thymidylate biosynthesis and DNA replication (Carreras and Santi, 1995). The methylation reaction that TS catalyzes provides the sole *de novo* source of thymidylate (dTMP, 2′-deoxythymidine-5′-monophosphate). TS is a major target for cytotoxic agents, some

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of which are established anticancer drugs. TS-targeting drugs are inhibitors that bind at the active site and that resemble either the substrate 2′-deoxyuridine-5′-monophosphate (dUMP), being thus classified as dUMP binding-site inhibitors, or the cofactor 5,10-methylene-5,6,7,8-tetrahydrofolate (mTHF), thus named folate binding-site inhibitors. 5-FU and capecitabine belong to the first class of dUMP binding site inhibitors. They are prodrugs of the nucleotide 5-fluoro-deoxyuridine 2′-monophosphate (FdUMP) that bind covalently and behave as suicide inhibitors. Drugs of the second class include methotrexate (MTX), the well-established pioneer high affinity inhibitor of dihydrofolate reductase (DHFR), behave as competitive/near competitive inhibitors and are very similar to reduced folate antagonists. Pemetrexed (PMX; Alimta), raltitrexed (Tomudex) and pralatrexate are structural folate antagonists of this class currently in use as antineoplastic agents. It should be emphasized that these antifolates display markedly enhanced inhibitory activity of TS in their polyglutamylated form (Ward et al., 1992; Shih et al., 1997). Nowadays, TS-based therapies can be defined as targeted therapies on the basis of their cellular inhibition mechanisms. However, PMX, raltitrexed and pralatrexate are known to inhibit TS as their primary drug target or just one of the targets, their multi-target activity being now well-established. The main drawback of these therapies is related to drug toxicity and the development of drug resistance, a phenomenon that may be multifactorial in nature but is often related to the overexpression of TS and some other folate-dependent proteins. Thus, alternative strategies to target TS are presently being pursued (Gonen and Assaraf, 2012; Garg et al., 2010).

The determination of medicinal chemists and pharmacologists of the role of TS as a prime target for anticancer chemotherapy is changing. While a former, reductionist view assumed TS as the therapeutic target, according to a more recent, holistic view, it is a key protein within a complex network that involves other proteins as well as nucleic acids. We further change the concept from network to netwire, as this latest definition evolves from the pathway/network concept by including feedback regulation and recycling of information, and is thus more suitable to account for what occurs in cellular metabolism. In fact, a drug delivered into a cell gives rise to a modulation of a broad set of proteins, nucleic acids and other biomolecules, with feedback effects observed, as in the case of DHFR, TS and various other enzymes (Hyduke and Palsson, 2010). A classic example is provided by the autoregulation of target-protein expression through direct interaction of the TS protein with its own mRNA (Chu et al., 2003). The netwire concept lays on the polypharmacological basis. According to this approach, more targets are necessarily involved in the description of the complex mechanism that governs the pharmacological activity of protein-targeting drugs. The multiple-target interactions of TS-targeting drugs are well known to biologists, pharmacologists, and clinical scientists. To provide just one example, PMX is known to target at least three proteins, TS, GARFT, and DHFR, known as folate-dependent proteins (Exinger et al., 2003). Albeit, the inhibition of TS by PMX is by far more dominant than DHFR and GARFT especially when PMX is polyglutamylated (Gonen and Assaraf, 2012).

In the present paper, we review the molecular aspects of the activity of TS-targeting anticancer drugs, discuss how such mechanisms are related to clinical drug resistance. Finally, we discuss the possibilities to overcome drug resistance using a molecular mechanistic approach based on recent studies. The entire review is tightly focused on the importance of the modulations of metabolic pathways, their analysis and the assessment of their putative roles within all the netwires involved using a meta-analysis approach.

2. Thymidylate synthase: structural insights

A structural description of the TS protein is important to understand how the inhibitors bind and how protein–inhibitor complex formation is influencing the functionality of the enzyme at the cellular level. Among ligands, both substrates and inhibitors show well-recognized binding sites and form TS-ligand complexes that induce specific biological responses at the cellular level.

TS is a homodimer with two active sites, each formed by residues from both monomers. In each monomer there are two main domains, a larger, conserved domain (residues 1–98 and 130–313) and a smaller, variable one (the small domain, residues 99–129) (Fig. 1A). Each monomer shows α- and β-fold (α + β). A five stranded β-sheet in each large domain forms the dimer interface (Fig. 1B). Forty-five residues of each monomer interact with residues of the opposite monomer. Among these, we have identified some key residues that are important for the monomer–monomer interaction and dimer stability (Cardinale et al., 2010). Cys195 in the catalytic loop (residues 184–199) is the catalytic amino acid that reacts with carbon C6 of dUMP, hence forming a covalent complex. Other important regions that can be recognized in the enzyme structure are the loop at the interface (residues 144–158), the loop around Arg50 (residues 47–54) and the C-terminal region (residues 308–313) (Fig. 1C) (Ferrari et al., 2008). The TS homodimer has been observed in two different forms, one active and one inactive. In the inactive form, the catalytic loop is twisted so that Cys195 is no longer directed into the active site and available for substrate binding (Phan et al., 2001b; Salo-Ahen and Wade, 2011). Several different binding sites are known for TS as shown by the high number of X-ray crystal structures of TS-inhibitor complexes existing in the PDB: the substrates, dUMP and folates, and their analogs such as dUMP and folate related inhibitors (*i.e.* antifolates), bind near the active site in the catalytic pocket (Phan et al., 2001a). In the ternary complex with TS (PDB ID: 1HVY), the uracil ring of dUMP forms a covalent bond with the catalytic Cys195 and two hydrogen bonds with the side-chain of Asn226 and the backbone of Asp118. The ribose moiety forms two hydrogen bonds with His256 and Tyr258 and interacts with Ser216 and Asp218. The phosphate moiety forms hydrogen bonds with Ser216 and four arginine residues, Arg50, Arg215, Arg175′, Arg176′, the last two residues belonging to the opposite monomer. Active-site bound dUMP forms the floor for an ordered and sequential binding of the folate cofactor and its analogs and the release of the products (Finer-Moore et al., 2003; Stroud and Finer-Moore, 2003). The X-ray crystal structure of two folate analogs with TS are known: raltitrexed and PMX (Fig. 2A). They have been shown to bind TS in such a way that: (i) the quinazolinone and the pyrrolo[2,3- *d*]pyrimidinone rings lay over dUMP; (ii) the aminothiophene-carbonyl and the benzoyl moieties interact with Glu87, Ile108, Trp109, Leu221 and Phe225; (iii) the glutamic tails are addressed toward the entrance of the active site, interact with Phe80, Leu221, and form hydrogen bonds with water molecules (Figs. 1B and 2A). The binding sites of other folate analogs (such as methotrexate) have been studied with TS isolated from other organisms (PDB IDs: 3K2H, 1AXW) and all share the same binding site.

TS is an mRNA binding protein which auto-regulates its own levels (Chu et al., 2003). It has been suggested that residues within the interface region are involved in the binding of TS with its mRNA. Indeed, peptides corresponding to the TS amino acid sequence residues 31–47, 56–72, 131–147, 176–192, 201–217 and containing interface residues bind TS mRNA (Voeller et al., 2002) (Fig. 1D).

Finally, some octapeptides have been shown to bind at the monomer–monomer interface, in a region near the catalytic loop (Cardinale et al., 2011; Tochowicz et al., 2015). Their binding