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Differential 3-bromopyruvate inhibition of cytosolic and mitochondrial human serine hydroxymethyltransferase isoforms, key enzymes in cancer metabolic reprogramming☆

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ABSTRACT

The cytosolic and mitochondrial isoforms of serine hydroxymethyltransferase (SHMT1 and SHMT2, respectively) are well-recognized targets of cancer research, since their activity is critical for purine and pyrimidine biosynthesis and because of their prominent role in the metabolic reprogramming of cancer cells. Here we show that 3-bromopyruvate (3BP), a potent novel *anti*-tumour agent believed to function primarily by blocking energy metabolism, differentially inactivates human SHMT1 and SHMT2. SHMT1 is completely inhibited by 3BP, whereas SHMT2 retains a significant fraction of activity. Site directed mutagenesis experiments on SHMT1 demonstrate that selective inhibition relies on the presence of a cysteine residue at the active site of SHMT1 (Cys204) that is absent in SHMT2. Our results show that 3BP binds to SHMT1 active site, forming an enzyme-3BP complex, before reacting with Cys204. The physiological substrate L-serine is still able to bind at the active site of the inhibited enzyme, although catalysis does not occur. Modelling studies suggest that alkylation of Cys204 prevents a productive binding of L-serine, hampering interaction between substrate and Arg402. Conversely, the partial inactivation of SHMT2 takes place without the formation of a 3BP-enzyme complex. The introduction of a cysteine residue in the active site of SHMT2 by site directed mutagenesis (A206C mutation), at a location corresponding to that of Cys204 in SHMT1, yields an enzyme that forms a 3BP-enzyme complex and is completely inactivated. This work sets the basis for the development of selective SHMT1 inhibitors that target Cys204, starting from the structure and reactivity of 3BP.

1. Introduction

3-Bromopyruvate (3BP), a pyruvate analogue and a strong alkylating molecule, has proven to be a powerful anticancer agent [1] and has been recently accepted for Phase I clinical evaluation in patients with primary and metastatic liver cancer (source: prescienceLabs.com). 3BP has been shown to abolish cell ATP production via inhibition of both glycolysis and oxidative phosphorylation [2], inducing rapid cancer cell death in several animal tumours, including hepatocarcinomas, colon and breast cancers [3–6]. Because 3-BP is very reactive and is well known as a non-specific thiols alkylating agent (Scheme 1), it seems unlikely to represent a novel candidate anti-cancer drug, since most chemotherapeutic agents are tailored for specific targets and are relatively non-reactive. As a matter of fact, 3BP is known to affect several targets involved in cellular energy metabolism, in glycolysis and in mitochondrial oxidative phosphorylation, such as hexokinase-II, glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase, succinate dehydrogenase and vacuolar ATPase [7–9]. These data and the non-specific alkylating properties of 3BP raise the question as to whether 3BP may exert its antitumor effect via reactions with as yet undetermined targets, such as those involved in the high needs of tumour cells for anabolic building blocks.

Serine hydroxymethyltransferase (SHMT) (EC 2.1.2.1) is a pyridoxal phosphate-dependent enzyme that catalyses the conversion of serine and tetrahydrofolate (H_4PteGlu) into glycine and 5,10-methylenetetrahydrofolate [10]. In the human genome, two genes encoding SHMT are found, expressing a cytosolic (SHMT1) and a mitochondrial (SHMT2)

Abbreviations: SHMT, serine hydroxymethyltransferase; SHMT1, cytosolic SHMT; SHMT2, mitochondrial SHMT; 3BP, 3-bromopyruvate; PLP, pyridoxal 5′-phosphate; H_4PteGlu, tetrahydropteroylglutamate.

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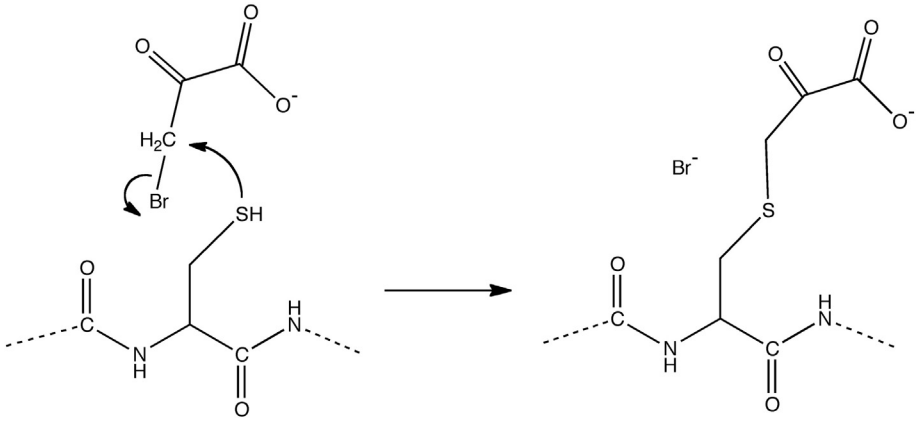

Scheme 1. Reaction between 3-bromopyruvate and a cysteine residue. Cysteine residues react with 3BP in a nucleophilic substitution in which a bromide ion is eliminated and a pyruvylated cysteine is formed.

isoform [11]. While in the cytosol serine uptake and the carbon units of H_4PteGlu are mainly needed for nucleotide and methionine biosynthesis [12], the activation of mitochondrial one-carbon metabolism is also required to produce NADPH [13,14]. Moreover, the mitochondrial production of glycine can fuel heme biosynthesis and therefore oxidative phosphorylation [15]. Therefore, SHMT isozymes occupy a critical position at the convergence of two key pathways for chemotherapeutic intervention: serine/glycine metabolism and nucleotide biosynthesis [16]. Accordingly, changes in the expression or activity of SHMT have been shown in several tumours. In particular, SHMT2 is known to play a crucial role in the serine/glycine metabolism of several cancer cell types, including colon and breast [17], while SHMT1 is overexpressed in tumour-initiating lung cancer cells [18] and in patient-derived lung cancer tissue samples [19]. These observations suggest that a specific inhibition of either SHMT1 or SHMT2 may be a successful therapeutic strategy against different tumours.

For several decades, SHMT has been proposed as a target in cancer chemotherapy research. Methotrexate and 5-fluorouracil, which are among the oldest antimetabolites still routinely used as anticancer agents, exert their action by targeting enzymes that, like SHMT, are in the pathway leading to 2′-deoxythymidine monophosphate (dTMP) [20,21]. Nevertheless, only a few studies focused on drug design strategies for the inhibition of SHMT. The only antifolate compounds with anticancer activity found to inhibit SHMT, apparently irreversibly, were the quite toxic sulfonyl fluoride triazine derivatives [22]. Leucovorin (5-formyl-H_4PteGlu) has been indicated as another inhibitor of SHMT [23,24]. However, after administration, leucovorin is readily converted into folate derivatives. The search for selective serine analogues as SHMT inhibitors has been much less successful [25]. More recently, our research group identified Pemetrexed [26], Lometrexol [27] and the pyrazolopyran derivative 2.12 [28] as weak inhibitors of the human SHMT1 enzyme. A detailed, comparative characterization of the structural, functional and inhibition properties of SHMT1 and SHMT2 is required to unravel similarities and differences between the two isozymes that may be exploited in the design of specific inhibitors.

Some work on 3BP and similar halogenated alkylating agents had already been carried out 40 years ago on both isoforms of SHMT, but from non-human sources. 3BP, chloroacetaldehyde, and iodoacetamide were found to inactivate rabbit liver SHMT1, but none of them inactivated SHMT2, although the basis of this inactivation was not unravelled [29]. In a slightly more recent study, it was shown that iodoacetate inactivated rabbit liver apo-SHMT1 (but not holo-SHMT1) by alkylation of a cysteine residue (Cys204) that was postulated to be at the active site. In this investigation, 3BP was not taken into consideration [30].

From scrutiny of SHMT1 crystal structural, it can be noticed that Cys204, which is actually located at the active site of SHMT1, is not conserved in SHMT2, where it is replaced by an alanine residue. In the present study, the role of cysteine residues in the mechanism of selective 3BP inactivation of human SHMT isoforms was investigated.

2. Materials and methods

2.1. Materials

3-Bromopyruvic acid, DL-threo-3-phenylserine and all other amino acids were purchased from Sigma-Aldrich. (6*S*) H_4PteGlu, was a gift from Merk & Cie, Schaffhausen, Switzerland. All other reagents were from Sigma-Aldrich. Recombinant cytosolic and mitochondrial SHMT isozymes were expressed in *Escherichia coli* and purified as previously described [31,32]. Inclusion of 5 mM mercaptoethanol in the inhibition reactions completely protected both isozymes from inactivation (data not shown). Therefore, mercaptoethanol was added to all buffers during the purification procedure, but it was left out in the final dialysis step to avoid interference with 3BP reactions. Methylene tetrahydrofolate dehydrogenase was recombinantly expressed and purified as described in [33]. Apo-SHMT1 was prepared from the holo-form of the enzyme using L-cysteine as previously described [34]. The subunit concentration of holo- and apo-SHMT1 were calculated according to molar absorptivity values of $\varepsilon_{280} = 47{,}565\ cm^{-1}\ M^{-1}$ and $\varepsilon_{280} = 39{,}310\ cm^{-1}\ M^{-1}$, whereas a values of $\varepsilon_{280} = 43{,}887\ cm^{-1}\ M^{-1}$ was used for holo-SHMT2 [32].

2.2. Site-directed mutagenesis

The SHMT1 and SHMT2 mutant forms were produced by site-directed mutagenesis using the related pET22b(+)::SHMT plasmids as template DNA [32] and the Quick-Change™ method from Stratagene (La Jolla, CA). Two complementary oligonucleotides, synthesized by MWG-Biotech AG (Anzinger, Germany) containing the mutations were used as primers for each mutagenesis reaction (only 5′ to 3′ primers are reported): SHMT1 C110A 5′-GCTGGACCCACAGGCCTGGGG GGTCAACG-3′, SHMT1 C204A 5′-CGCAGGAACCAGCGCCTACTCCCGAA ACC-3′ and SHMT2 A206C 5′-GCTGGCACCAGCTGCTATGCTCGCCTC-3′ (mutated bases are underlined). The double SHMT1 C110A/C204A mutant was obtained introducing the C110A mutation in the C204A DNA template. *E. coli* DH5α cells were transformed and used to amplify the mutated plasmid. Both strands of the coding region of the mutated genes were sequenced. The only differences with respect to wild type were those intended. Expression of the mutant proteins was carried out in the BL21 (DE3) *E. coli* strain and purification performed as described for the wild type enzymes.