

Short communication

TRANSPORT OF 3-BROMOPYRUVATE ACROSS THE HUMAN ERYTHROCYTE MEMBRANE

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Abstract: 3-Bromopyruvic acid (3-BP) is a promising anticancer compound because it is a strong inhibitor of glycolytic enzymes, especially glyceraldehyde 3-phosphate dehydrogenase. The Warburg effect means that malignant cells are much more dependent on glycolysis than normal cells. Potential complications of anticancer therapy with 3-BP are side effects due to its interaction with normal cells, especially erythrocytes. Transport into cells is critical for 3-BP to have intracellular effects. The aim of our study was the kinetic characterization of 3-BP transport into human erythrocytes. 3-BP uptake by erythrocytes was linear within the first 3 min and pH-dependent. The transport rate decreased with increasing pH in the range of 6.0–8.0. The K_m and V_m values for 3-BP transport were 0.89 mM and 0.94 mmol/(l cells x min), respectively. The transport was inhibited competitively by pyruvate and significantly inhibited by DIDS, SITS, and 1-cyano-4-hydroxycinnamic acid. Flavonoids also inhibited 3-BP transport: the most potent inhibition was found for luteolin and quercetin.

Keywords: 3-Bromopyruvic acid, Erythrocytes, Polyphenols, Flavonoids, Transport, Monocarboxylate transporter, 1-Cyano-4-hydroxycinnamic acid, Luteolin, Quercetin

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Abbreviations used: 3-BP – 3-bromopyruvate; CHC – 1-cyano-4-hydroxycinnamic acid; DIDS – 4,4'-diisothiocyanostilbene-2,2'-disulphonate; MCT – monocarboxylate transporter; SITS – 4-acetamido-4'-isothiocyanostilbene-2,2'-disulphonic acid

INTRODUCTION

It has been estimated that about 400,000 cancer patients die each year due to the induction of multidrug resistance to chemotherapy. This indicates a clear need to find new chemotherapeutics. Preferential killing of cancer cells without significant toxicity to normal cells, based on the biochemical differences between them, is one of the most important considerations in cancer chemotherapy.

In most normal cells, at least 90% of adenosine triphosphate (ATP) production is from mitochondrial oxidative phosphorylation, while tumor cells are approximately 50% dependent on cytoplasmic, aerobic glycolysis, which is a tumor-specific feature usually termed the Warburg effect [1]. New generations of glycolytic inhibitors with good safety profiles and high potency and stability are an important focus in the search for less toxic cancer treatments. 3-bromopyruvate (3-BP) is a very promising compound in this respect.

Initial studies showed that 3-BP is effective at eliminating aggressive liver tumors and proved that it has a higher cell-killing capacity than 2-deoxyglucose in a hepatoma VX2 model [2]. 3-BP was also tested in several cancer cell lines [3–5]. Its effectiveness has been proven in animal experiments, and one case of clinical treatment has been reported [2, 6–8]. Importantly, as a polar compound, 3-BP is not a substrate for the PDR network in yeast [9] or the MDR transporters in mammalian cells [10].

A crucial problem in the future clinical application of 3-BP is the prevention of its possible side effects. Geschwind et al. found that 3-BP showed minimal cytotoxicity to normal liver cells at a low concentration (0.5 mM) [11]. It has been reported that the portal veins, the sinusoids, and the bile ducts in the normal liver remained completely intact, with the only apparent damage found in the peribiliary arteriolar complexes. That damage was only present in some samples and at much higher concentrations (5 mM 3-BP) [11].

The toxicity of intra-arterial delivery of 3-BP at various concentrations has also been specifically investigated using a normal rabbit model. It was found that selective intra-arterial administration of 25 μ M 3-BP can cause considerable toxicity, not only in the liver but also in the gastrointestinal system. This toxicity is dose dependent, with death occurring at high doses [12]. The capacity of 3-BP to inhibit energy production in the fractions obtained from normal cells (rat hepatocytes) and in isolated rat thymocytes has also been studied. The findings were that the main targets of the drug were glyceraldehyde 3-phosphate dehydrogenase (not hexokinase, as suggested for hepatoma cells) and succinate-driven ATP synthesis [13].

We found that exposure of erythrocytes to concentrations between 0 and 2 mM 3-BP did not affect the osmotic fragility of the erythrocytes. However, treating erythrocytes with 3-BP induced a loss of both GSH and GSSG, which indicates a decrease in the total glutathione level and an increase in the GSSG/GSH ratio. This is evidence of oxidative stress. The activities of superoxide dismutase (SOD) and glutathione *S*-transferase (GST) were significantly decreased by 3-BP [14].

Transport into the cell is critical for the intracellular action of 3-BP [15]. In mammals, the main mediators are expected to be the monocarboxylate transporters (MCTs). Permeation of 3-BP into the erythrocytes, which are present in huge amounts in the bloodstream, is of considerable interest because:

1. It decreases the effective pool of 3-BP that can reach the target cells during infusion.
2. It determines the side effects of 3-BP from the interaction of this compound with erythrocytes.

Our aim was the kinetic characterization of 3-BP transport into human erythrocytes. In particular, we intended to evaluate the pH dependence of 3-BP transport, to characterize the kinetics of the transport and to determine the effects on 3-BP transport into human erythrocytes of various potential inhibitors, such as DIDS (4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid), SITS (4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonate), CHC (1-cyano-4-hydroxycinnamic acid), and various natural compounds, especially polyphenols (quinic acid, naringin, ferulic acid, elagic acid, daidzein, apigenin, luteolin, genistein, quercetin, kaempferol, caffeic acid, and resveratrol).

MATERIALS AND METHODS

Reagents and polyphenols

3-BP and all of the other reagents were obtained from Sigma, except for phosphate-buffered saline (PBS: 145 mM NaCl, 1.9 mM NaH₂PO₄, 8.1 mM Na₂HPO₄), which was from Lab Empire. (¹⁴C)-3-BP (15 mCi/mmol) was donated by Young H. Ko. The polyphenols quinic acid, naringin, ferulic acid, elagic acid, daidzein, apigenin, luteolin, genistein, quercetin, kaempferol, caffeic acid, and resveratrol were purchased from Santa Cruz Biotechnology, Inc.

Transport of 3-BP

Transport of 3-BP was studied using a radiolabeled substrate, (¹⁴C)-3-BP. Human blood was obtained from a healthy female volunteer, aged 35. The study was approved by the local Ethical Committee (the Regional Medical Council, Rzeszow). The erythrocytes were washed and suspended in PBS at a hematocrit of 50%. For transport measurements, erythrocytes were added (as the last component) to appropriate media containing 6.7 μM (¹⁴C)-3-BP, appropriate concentrations of non-radioactive 3-BP (3.3 μM – 2 mM) and other additives (if present). Transport inhibitors were added to a final concentration of 100 μM from stock 10 mM solutions in PBS or DMSO, except for the studies of the concentration dependence of the inhibition (2.5–100 μM). The final concentration of erythrocytes was 5% and that of DMSO (if added) was 1%. The samples were vortexed immediately. After incubation for 60 s at room temperature, 500-μl aliquots were centrifuged through a layer of dibutyl phthalate (1 ml) in an Eppendorf tube (15 s, 13500 rpm).

The experiments were performed at ambient temperature ($21 \pm 1^\circ\text{C}$). The erythrocyte sediment was quickly washed twice with ice-cold PBS. Then, 100 μl of 1 M NaOH was added and the whole was incubated overnight. Next, 30 μl of 30% H_2O_2 was added, and after incubation for 2 h, 3 ml of a scintillation cocktail consisting of 4.0 g/l 2,5-diphenyloxazole and 0.1 g/l 1,4-bis(5-phenyl-2-oxazol)benzene in dioxane was added. The samples were counted for radioactivity in a 1219 Rackbeta Liquid Scintillation Counter (LKB Wallac).

Statistical analysis

Each result is based on 3 parallel measurements. The data are presented as the means \pm SD. The statistical significance of differences between the means was estimated using the Kruskal-Wallis test. Tukey's post hoc test was used to compare the mean activities or levels of the studied parameters with 5 different threshold activities or levels for those parameters. The statistical analysis of the data was performed using STATISTICA, version 10 (www.statsoft.com).

RESULTS

The amount of 3-BP accumulated in the erythrocytes was a linear function of time during the initial 3 to 5 min (not shown). We chose 1 min as the time for routine measurements. In order to check whether the pH dependence of 3-BP transport is in line with what is typical for monocarboxylate transporters, the transport rate in the pH range of 6.0–8.0 was assessed. We found that it decreased with increasing pH (Fig. 1). Routine measurements were performed at physiological pH (7.4).

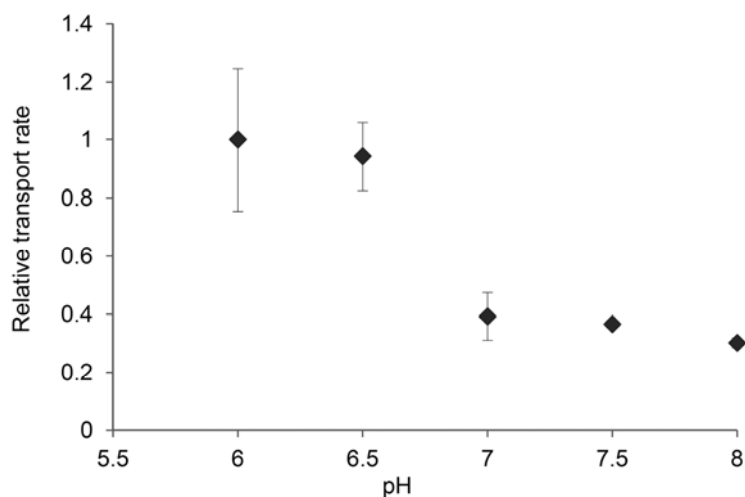


Fig. 1. Dependence of 3-BP transport on pH. The uptake of 10 μM 3-BP was measured in physiological saline buffered to different pH (6.0–8.0) at room temperature for 1 min.

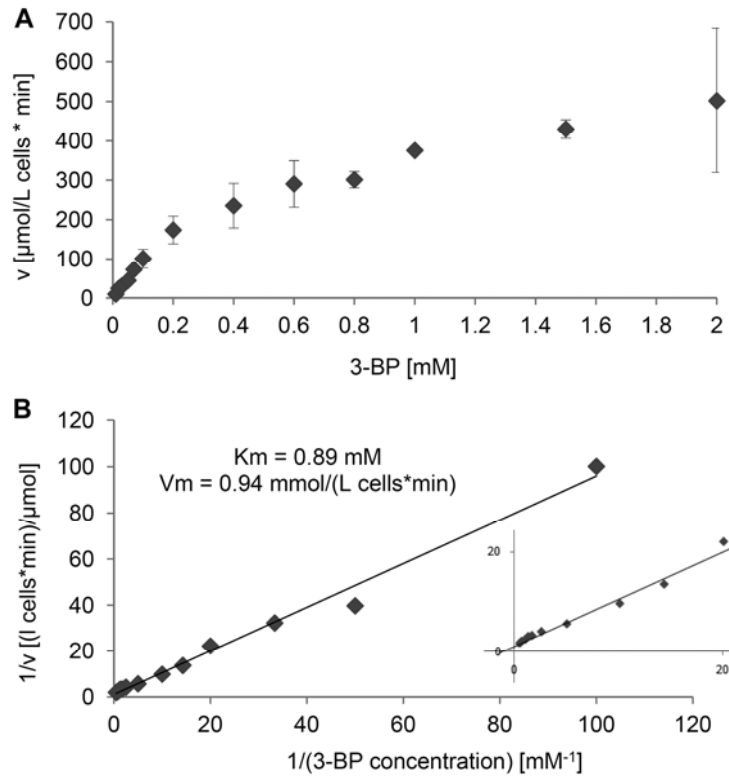


Fig. 2. Dependence of the 3-BP transport rate into erythrocytes on the 3-BP concentration in the extracellular medium. A – Michaelis-Menten plot. B – Lineweaver-Burk plot.

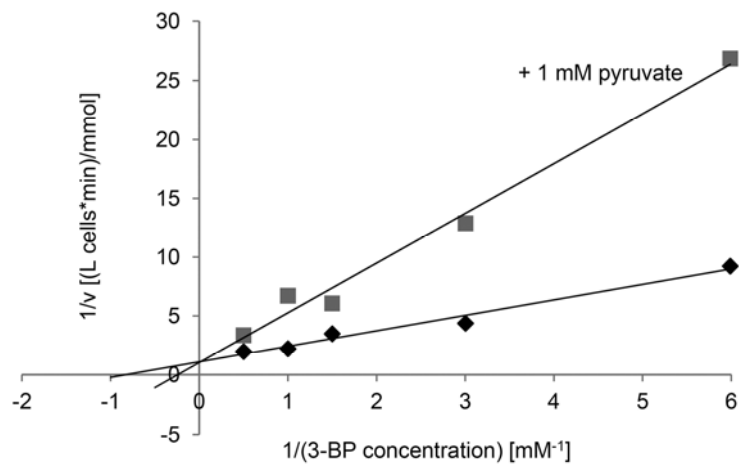


Fig. 3. Competitive inhibition of 3-BP transport by pyruvate. 3-BP uptake was measured in the absence and presence of 1 mM pyruvate for various concentrations of 3-BP.

The transport followed Michaelis-Menten kinetics with an apparent K_m value of 0.89 ± 0.22 mM and a V_m value of 0.94 ± 0.02 mmol/(1 cells \times min), as shown in Fig. 2. The transport was inhibited competitively by pyruvate (Fig. 3), which is compatible with the idea that 3-BP shares the same transporter(s) with pyruvate. For the set of data shown in Fig. 4, the apparent K_m /apparent V_m values in the absence and in the presence of pyruvate were 0.99 and 4.09 mM, respectively. From these data, a K_i value of 0.32 mM for pyruvate was calculated.

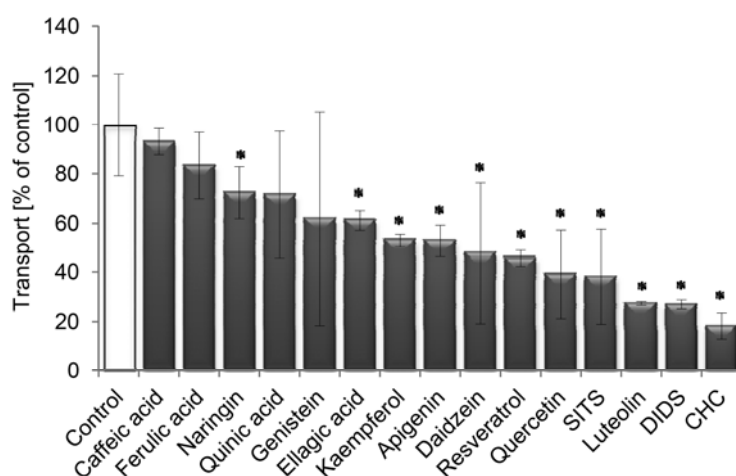


Fig. 4. Inhibition of 3-BP transport by various compounds. All of the transport modulators were added to a final concentration of 100 μ M. 3-BP concentration was 10 μ M. * $p < 0.05$ (relative to the control).

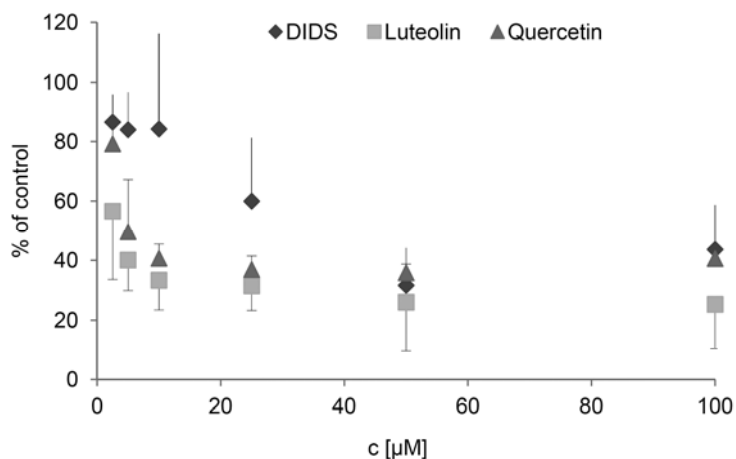


Fig. 5. Comparison of the effect of DIDS, luteolin and quercetin on the rate of 3-BP transport into human erythrocytes. The 3-BP concentration was 10 μ M.

The transport of 3-BP was inhibited by MCT and the band 3 inhibitors DIDS and SITS [16], and by the more specific MCT inhibitor CHC (Figs 4 and 5). Interestingly, the transport was also inhibited by several natural polyphenols, including luteolin, quercetin, resveratrol, daidzein, apigenin, kaempferol, ellagic acid, and naringin (Fig. 4). Luteolin was the most potent inhibitor of the compounds studied (Fig. 4), with a c_{50} of about 3 μ M (Fig. 5).

We checked whether 3-BP bound to proteins inside erythrocytes by precipitating proteins in an erythrocyte suspension with 10% trichloroacetic acid (TCA) immediately after the uptake measurement and counting the radioactivity of the protein sediment and the supernatant. A negligible fraction of the radioactivity (less than 1%) was associated with the protein sediment after 1 min of contact with the radioactive probe at concentrations of both 10 μ M and 1 mM, with the cells showing that during this time, 3-BP remained either unbound or bound to low-molecular weight compounds that were not precipitated with TCA (presumably glutathione).

DISCUSSION

The anticancer efficacy of the pyruvate analog 3-BP has been demonstrated in multiple tumor models [7]. Data obtained from tissue-autoradiography of rats infused with 14 C-3-BP showed a strong 14 C signal in the tissue sections of various organs except the brain, corroborating that 3-BP does not cross the blood-brain barrier [23]. A fundamental aspect determining the cellular actions of 3-BP is its transport into the cells, which is mainly dependent on monocarboxylate transporters (MCTs).

Hypoxia and oncogene expression both stimulate glycolytic metabolism in tumors, leading to lactate production. Two control points regulate lactate shuttles: the lactate dehydrogenase-dependent conversion of lactate into pyruvate (and back), and the transport of lactate into and out of cells by specific MCTs. These constitute a family of 14 transporters that are also known as solute carrier 16 (SLC16) proteins. They carry single-carboxylate molecules across biological membranes [17].

Four members of the MCT family (MCT1-MCT4) have been described to be proton-linked MCTs. MCT1 and MCT4 are the two major MCTs expressed in tumor cells and they are promising targets for therapy. In fact, of the 20,000 mRNAs examined, the mRNA levels of the SLC16A1 gene, coding for MCT1, were the best single predictor of 3-BP sensitivity.

MCT1 expression correlates with elevated glycolysis, and it may be possible to enhance the efficacy of 3-BP by concomitant treatment with glycolytic inhibitors so as to exploit the high glycolytic demand of tumors and the cancer-enriched expression of MCT1 [18]. The MCT4 transporter was also demonstrated to participate in 3-BP transport into some types of cancer cells [5]. MCT1 is the most widely expressed MCT. It plays an active role in the uptake of lactate into the heart, skeletal muscle, erythrocytes, and liver [19]. MCT1 inhibition allows

the simultaneous disruption of metabolic cooperativity and angiogenesis in cancer with the same agent, opening a new path for novel anticancer therapies [20]. MCT4 is primarily expressed in highly glycolytic cells, such as white muscle fibers, and is upregulated in response to hypoxia [21]. Translating the knowledge of lactate shuttles to the cancer field offers new possibilities to therapeutically target the hypoxic tumor microenvironment and to tackle tumor angiogenesis [22].

MCT1 is present in erythrocytes and is the main transporter responsible for the transmembrane exchange of lactate and other monocarboxylic acids. Our results demonstrate that 3-BP is effectively transported into erythrocytes, apparently mainly by MCT1, as its transport is competitively inhibited by pyruvate. The pH dependence of 3-BP transport is typical for MCT. The K_m value for 3-BP is of an order of 1 mM, and the maximal transport rate is about 1 mmol/(l cells x min). The K_m value obtained is thus similar to that reported for pyruvate (0.7 mM) [23]. These values of K_m and V_m indicate that erythrocytes may be an important undesired sink during intravenous infusion of 3-BP. In the patented treatment (Patent application Nr. US 2003-0087961 A1, May 8, 2003; Nr. US 2007-0203074 A1, August 30, 2007; Nr. US 2010-0197612 A1, August 5, 2010), an effective dose of 3-BP administered intra-arterially is about 1 to 25 mM. It has been reported that 3-BP binds covalently to plasma proteins [24], but our data indicate that in spite of this effect, considerable amounts of 3-BP can be trapped inside erythrocytes over a short period, decreasing the effective 3-BP dose and potentially affecting erythrocyte functions [14].

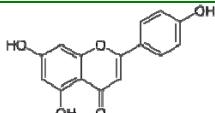
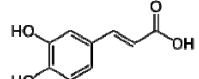
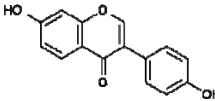
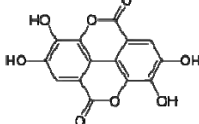
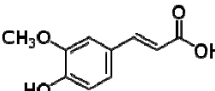
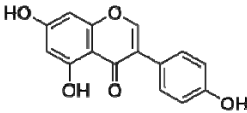
Cellular uptake of 3-BP can be modulated with MCT inhibitors. The known inhibitors of transport of MCT1 substrates include CHC, phenyl-pyruvate, niflumic acid, 4,4-diisothiocyanostilbene-2,2-disulfonate (DIDS) and *p*-chloromercuribenzenesulfonate. It has also been found that the flavonoids phloretin and quercetin effectively inhibit MCT1. Inhibition of lactic acid efflux from Ehrlich ascites cells with bioflavonoids such as quercetin been shown to decrease intracellular pH and thus to inhibit glycolysis and cell growth [19, 25]. The inhibition is mainly competitive and some flavonoids, especially epigallocatechin-3-gallate and (-)-epicatechin-3-gallate, are substrates for MCT [26]. Flavonoids are also known to inhibit various efflux transporters, including ABCB1 (P-glycoprotein), ABCC1 (MRP1), ABCC2 (MRP2), ABCG2 (breast cancer resistance protein) [27–30], and OATP influx transporters [31, 32]. Our data indicate that CHC, DIDS, SITS, and a range of polyphenols, especially flavonoids, inhibit the transport of 3-BP.

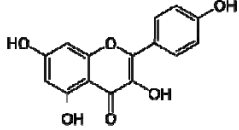
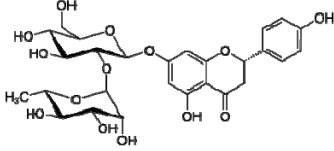
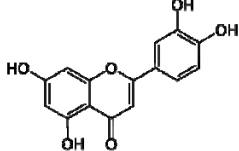
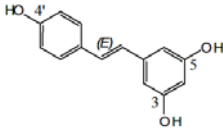
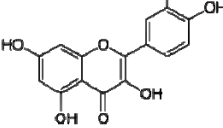
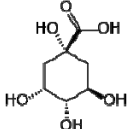
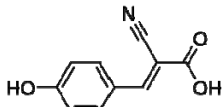
Wang and Morris [33] suggested that there are some structural elements that appear to be important for the MCT1-inhibitory activity of flavonoids. The presence of a 4'-hydroxy group appears to moderately increase the inhibitory effect (for example, the inhibitory effect of apigenin is greater than that of chrysin), whereas methylation of this hydroxyl group decreases the inhibitory activity (for example, diosmetin is much less potent than luteolin). The addition of another hydroxyl group at position 3' or 2' increases the inhibitory effect

(for example, luteolin is more potent than apigenin and morin is more potent than kaempferol), whereas the addition of a 5-hydroxyl group on ring A has minimal effect on the inhibitory effect (for example, fisetin has an inhibitory effect similar to that of quercetin). Luteolin and quercetin were especially effective effectors, comparable to or even more potent than DIDS (Fig. 5). CHC was the most potent inhibitor of 3-BP transport (Fig. 4).

Our results concur with these conclusions with respect to 3-BP transport. Our data suggest that neither the total number of –OH groups nor the number of phenolic groups in the molecule correlate with the inhibitory potency of the compounds studied. The –OH group at position 3 decreases the inhibitory effect: luteolin is a stronger inhibitor than quercetin or kaempferol. In general, flavonoids with an unsubstituted position 3 have a greater inhibitory potency (luteolin vs. genistein, quercetin and kaempferol). On the other hand, the presence of a second hydroxyl group in the B ring increases the inhibitory activity of flavonoids (luteolin vs. apigenin, quercetin vs. kaempferol). Resveratrol, which is not a flavonoid, but shares common structural elements with flavonoids, is also an inhibitor of 3-BP transport (Table 1).

Table 1. Structures of the phenols and polyphenols studied.

Total OH (phenolic OH)	Compound name	Structure
3 (3)	Apigenin	
1 (1)	Caffeic acid	
2 (2)	Daidzein	
4 (4)	Ellagic acid	
1 (1)	Ferulic acid	
3 (3)	Genistein	

Total OH (phenolic OH)	Compound name	Structure
4 (4)	Kaempferol	
8 (2)	Naringin	
4 (4)	Luteolin	
3 (3)	Resveratrol	
5 (5)	Quercetin	
4 (0)	Quinic acid	
1 (1)	1-cyano-4-hydroxycinnamic acid (CHC)	

It is known that hydrophobic monocarboxylates are substrates for MCT1. However, they interact strongly with the transporter and are difficult to release, so they act as transport inhibitors [24]. Comparing the inhibitory properties of such acids, flavonoids, and other compounds of similar structure may provide clues about the binding site of the transporter and sites of interaction with potential substrates.

Apart from MCT1, the band 3 protein (AE1, SLC4A1) is an erythrocyte membrane protein able to transport monocarboxylic acids, such as lactate, and can also be expected to transport 3-BP [34]. However, the contribution of band 3 to lactate transport has been reported to be minor in human erythrocytes [35].

Although flavonoids were reported to affect transport via band 3 [36], to the best of our knowledge, no significant inhibition of transport via band 3 has been reported, and the inhibitory action of flavonoids is apparently due to their effect on MCT1.

The inhibition of monocarboxylate transport by a range of natural polyphenols, some of them effective at low micromolar concentrations, is of particular interest. Such compounds can be ingested as part of the normal diet and as nutraceuticals to provide a protection against the side effects of antitumor therapy. Their possible effects on the uptake of 3-BP should be considered. On the one hand, they may hamper drug permeation of target cells. On the other hand, they may provide protection of normal cells against 3-BP, depending on their distribution in different cell types.

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