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Effect of sulfonamides as carbonic anhydrase VA and VB inhibitors on mitochondrial metabolic energy conversion

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ABSTRACT

Obesity is quickly becoming an increasing problem in the developed world. One of the major fundamental causes of obesity and diabetes is mitochondria dysfunction due to faulty metabolic pathways which alter the metabolic substrate flux resulting in the development of these diseases. This paper examines the role of mitochondrial carbonic anhydrase (CA) isozymes in the metabolism of pyruvate, acetate, and succinate when specific isozyme inhibitors are present. Using a sensitive electrochemical approach of wired mitochondria to analytically measure metabolic energy conversion, we determine the resulting metabolic difference after addition of an inhibitory compound. We found that certain sulfonamide analogues displayed broad spectrum inhibition of metabolism, where others only had significant effect on some metabolic pathways. Pyruvate metabolism always displayed the most dramatically affected metabolism by the sulfonamides followed by fatty acid metabolism, and then finally succinate metabolism. This allows for the possibility of using designed sulfonamide analogues to target specific mitochondrial CA isozymes in order to subtly shift metabolism and glucogenesis flux to treat obesity and diabetes.

1. Introduction

Obesity can be considered the most common disease in the developed world. It directly causes other more serious diseases such as diabetes and cardiovascular disease.[1] There have been many weight loss drugs that have been developed to directly deal with obesity, but all of these drugs have risk of serious side effects.[2,3] This is because many are developed based on powerful mitochondrially active compounds such as dinitrophenol. Dinitrophenol (DNP) is a powerful uncoupling agent that essentially short circuits the mitochondrial proton pump and electron transport chain (ETC) by allowing protons to permeate the mitochondrial membrane so that mitochondrial metabolism continues unabated but does not generate any ATP.[4] The underlying idea for this type of treatment was to lower the ATP generation efficiency of the mitochondria so that more energy needed to be used to regenerate the same amount of ATP. The main problem with this type of treatment is that it is completely non-specific, so an organ's cells are affected as much as muscle cells and fat cells.[5–7]

Another example is the anti-obesity drug Phen-Fen marketed in the 1990s, which contained two active drugs compounds fenfluramine and phentermine that worked in concert to achieve fat loss.[8] Fenfluramine, a neuronal active compound, non-specifically targets the serotonin transporters causing the release of serotonin thereby decreasing hunger.[9] The main side effect of fenfluramine is cardiac valve damage due to the fenfluramine's ability to target serotonin releasing cells which are necessary for cardiac valve function. Phentermine is similar to the amphetamine class of compounds and acts by increasing catecholamine release.[10] It has the effect of both decreasing appetite and increasing heart rate. However, it has many side effects that are similar to other amphetamine compounds such as hypertension and anxiety.[11] When fenfluramine and phentermine are used together, the side effects of each become synergistic, so the result of combining these two compounds causes both hypertension and cardiac valve damage which can lead to serious incurable cardiovascular damage.[12]

These harsh drugs have claimed to be anti-obesity drugs, but they are more disruptive systemwide and often cause more serious problems than obesity does. Treatment for obesity should instead be more targeted and subtle to shift the metabolic pathways from generating excess glucose and resulting lipids. Obesity and type 2 diabetes are related diseases, because both the pancreas and adipose tissue are feedback regulated by one another.[13] If the mitochondrial metabolism is dysfunctional in organ cells, it causes the cells to not function properly resulting in one pathway being

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dominant over the other and the disease perpetuates. In order to shift this metabolic flux back to normal levels, we suggest that harsh mitochondrial toxins may not be the answer.

Mitochondrial carbonic anhydrases (CAs, EC 4.2.1.1) play a significant role in the metabolism of pyruvate, by accelerating the rate at which pyruvate carboxylase can convert pyruvate and bicarbonate into oxaloacetate.[14] This particular pathway only works with pyruvate, which is derived from glucose through glycolysis. This pathway is in equilibrium with the fatty acid pathway; therefore, if there is excess glucose, then the metabolic flux will be shifted towards pyruvate and away from fatty acids.[15,16] By using a specific mitochondrial CA inhibitor (CAI), it may be possible to shift the flux of mitochondrial metabolism towards using fatty acids and slow the metabolism of pyruvate.[14,15] Water reacts with CO_2 to generate bicarbonate and a proton under physiological pH at a highly diminished rate compared to the CA catalyzed reaction, so it will not stop the metabolic pathway of pyruvate through pyruvate carboxylase, but it will slow it down. We suggest that the rate at which pyruvate passes through the pathway will be slowed, so that fatty acid oxidation can become the dominant pathway, causing weight loss.

Sulfonamides are the most widely investigated class of CAIs as shown in Scheme 1, and acetazolamide (**AZA)** is clinically used for more than 50 year[14,16] . Other CAIs of sulfonamide and sulfamate type, such as zonisamide (**ZNS)** and topiramate (**TPM)** show a significant antiobesity effect (in obese epilepsy patients treated with such drugs) probably due to the mitochondrial CA inhibitory effects of these compounds.[14–16]

In this work we examine the effect of several CAIs (of types **1–5** as shown in Table 1)[17] on the mitochondrial metabolism of pyruvate, succinate, and short chain fatty acid. The sulfonamide analogue inhibitors used in this study have been shown to have very high activity and specificity for the inhibition of mitochondrial CA isozoymes hCA VA and hCA VB (h = human isoform) over other CAs found in other portions of cells and organs, such as the cytosolic hCA I and II.[17] Mitochondrial CAs are divided into two isoforms CA VA and VB, and have been indicated to be part of different pathways within the mitochondria.[14,15] There has been literature that suggests that each of these can be targeted with the proper compound[17], but analytical determination of mitochondrial metabolism with these inhibitors has not been examined. We suggest that by studying the electrochemistry of mitochondrial modified electrodes in a variety of substrates (i.e. pyruvate, fatty acids, etc.) to measure the output of the ETC, we can directly and quantitatively determine the level of effect on the metabolic pathways by the inhibitors. This could be used to screen these analogues to determine which ones will be better potential candidates for obesity treatment without the need for costly and time consuming cell or tissue culture experiments.

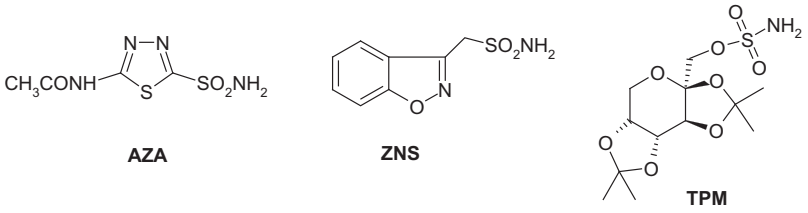
2. Materials and methods

2.1. Reagents

Sulfonamides **1–5** (as shown in Table 1) were synthesized as reported earlier by Supuran and co-workers.[17] All other reagents were used as received from Sigma–Aldrich.

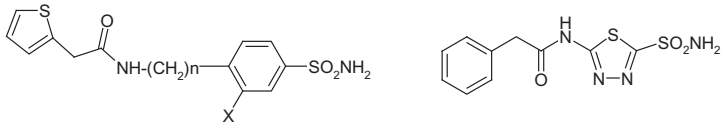
2.2. Mitochondrial extraction

Mitochondria were isolated from normal mice, C57BL/6, liver tissues according to Tappa et al.[18] as described in Reference.[19] In brief, liver tissues were homogenized in 0.33 M sucrose, 1.33 mM



Scheme 1. Structure of sulfonamide inhibitors in clinical use: acetazolamide (**AZA)**, zonisamide (**ZNS)** and topiramate (**TPM**).

Table 1
List of CAIs **1–5** and their activity against the mitochondrial CA isoforms



1-4 **5**

Compound	*n*	X	K_i[a] (nM)	
			hCA VA	hCA VB
1	0	–	7.2 ± 0.4	7.0 ± 0.6
2	0	Cl	7.7 ± 0.7	8.6 ± 0.5
3	1	–	9.1 ± 0.9	7.2 ± 0.7
4	2	–	10.2 ± 1.0	8.0 ± 0.7
5	–	–	8.4 ± 0.5	6.1 ± 0.4

Mean ± standard error, from three different assays.
[a] From ref.[17]

pH 7.4 Tris-EDTA containing 1 mM PMSF, 1 mM benzamidine and 5 mM iodoacetamide on ice. Liver homogenate was subjected to stepwise centrifugation. First, the liver homogenate was centrifuged at 500×*g* for 10 min, then the supernatant obtained was centrifuged once more at 500×*g* for 10 min. The supernatant after the second centrifugation was centrifuged at 20,000×*g* for 15 min. The mitochondrial pellet obtained was resuspended in pH 7.4 phosphate buffered saline (PBS). The protein concentration of the mitochondrial suspension was determined by micro Lowry's procedure using bovine serum albumin as a standard protein. The mitochondrial suspension was stored at −80 °C before use. The concentration of mitochondria suspended in PBS was determined as 18.6 mg/mL. Before use in fabricating mitochondrial electrodes, 1 mg/ml of ADP was added to the mitochondrial suspension.

2.3. Wiring of mitochondria onto electrodes

5.0 mg of COOH-modified multiwalled carbon nanotubes (OD 30–50 nm, L 1–10 μm, COOH-MWCNT) were added to a microcentrifuge tube. Then, 50 μL of ethanol was added to this vial to wet the nanotubes. To the same vial, 100 μL of de-ionized water was added and placed into a sonic water bath for 30 min to disperse the nanotubes. Once dispersed, the vial was placed in an icebath. After cooling, 100 μL of mouse mitochondria suspension was added to the vial and the vial was placed on a vortex mixer for 5–10 s. Immediately following the mixing, the mitochondria-MWCNT suspension was pipetted onto clean, polished glassy carbon electrodes (10 μL of mitochondria-MWCNT suspension per 0.07 cm^2 of electrode area). The electrodes were then placed under a small fan to accelerate drying. After 10 minutes, 1.5 μL of TBAB modified Nafion® (prepared as procedures in Reference[20,21]) suspension per 0.07 cm^2 of electrode area was pipetted on top of the electrode and placed back under the fan. Once dry, the electrodes were submersed for 1 h in a solution that contained 100 mM metabolic fuel (pyruvate, acetate, or succinate), 1 mg/ml ADP, 100 mM NaCl electrolyte, and 10 mM pH 8.0 phosphate buffer. The mitochondrial electrodes were used for electrochemical testing after soaking for 1 h. After the mitochondria modified electrodes were initially tested and characterized, they were soaked for 1 hour in substrate solution that contained a given CAI at 1 μM, and tested again to determine the effect on metabolism.

2.4. CA inhibition assay

An SX.18MV-R Applied Photophysics (Oxford, UK) stopped-flow instrument was used to assay the inhibition of various CA isozymes as reported by Khalifah.[22] The mitochondrial CA isozymes hCA VA and hCA VB used in the assay were recombinant proteins obtained as reported previously by the Supuran group.[23,24] 0.2 mM Phenol Red was used as an indicator at an absorbance of 557 nm with 10 mM HEPES (pH 7.4) as buffer and 0.1 M Na_2SO_4 or $NaClO_4$ (for maintaining constant the ionic strength; these anions are not inhibitory in the used concentration). The CA-catalyzed CO_2 hydration reaction was followed for a period of 5–10 s. Saturated CO_2 solutions in water at 25 °C were used as substrate. Stock solutions of inhibitors were prepared at a concentration of 10 mM (in DMSO–water 1:1, v/v) and serial dilutions of up to 0.01 nM were done with the assay buffer mentioned above. At least 7 different inhibitor concentrations were used for measuring the inhibition constant. Inhibitor and enzyme solutions were pre-incubated together for 10 min at room temperature prior to performing the assay, in order to allow for the formation of the E–I complex. Experiments were performed in triplicate experiments for each inhibitor concentration and the values reported throughout the paper are the mean of these results. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3 and the Cheng-Prosoff equation.

2.5. Electrochemical metabolic measurement

The mitochondria modified electrodes (anode) were placed into a beaker containing their respective substrate solutions, along with a platinum mesh cathode that was cleaned in concentrated nitric acid and was separated from the anolyte by a Nafion® NRE212 membrane which served as an ion selective salt bridge. The cathode compartment of the cell contained 10 mM pH 7.50 phosphate buffer with 100 mM $NaNO_3$ electrolyte. The setup is shown in Figure 1. The anode and cathode were connected to a CH Instruments model 810 potentiostat interfaced to a PC for electrochemical measurements. Each mitochondria modified electrode was allowed to reach maximum open circuit potential before performing a linear sweep voltammogram which started at the open circuit potential and ended at 0 V at a scan rate of 10 mV/s. All experiments were performed in triplicate and the reported uncertainties correspond to one standard deviation.

3. Results and discussion

CAIs showing potent inhibition of the mitochondrial CA isoforms, and also selectivity for CA VA/VB over CA I/II, have only recently been reported.[17] These highly lipophilic compounds incorporate phenyl(alkyl), halogenosubstituted-phenyl- or 1,3,4-thiadiazole-sulfonamide moieties and thienylacetamido; phenacetamido- and pyridinylacetamido tails. Compounds **1–5** investigated here belong to this sulfonamide type of CAIs and were shown to be low nanomolar hCA VA/VB inhibitors as shown in Table 1.[17] Table 1 also lists the inhibitor designations and structures. This research focused on studying their effect on mitochondrial function/mitochondrial metabolism to understand if they be effective as anti-obesity drugs. Recent research in our group has shown that electrochemical measurements of mitochondrial modified electrodes can be employed for studying mitochondrial function[25], because the mitochondrial modified electrodes have been shown to have the ability to measure ETC electron throughput by accepting electrons from cytochrome c before they can be accepted by cytochrome c oxidase, which normally reduces dioxygen to water

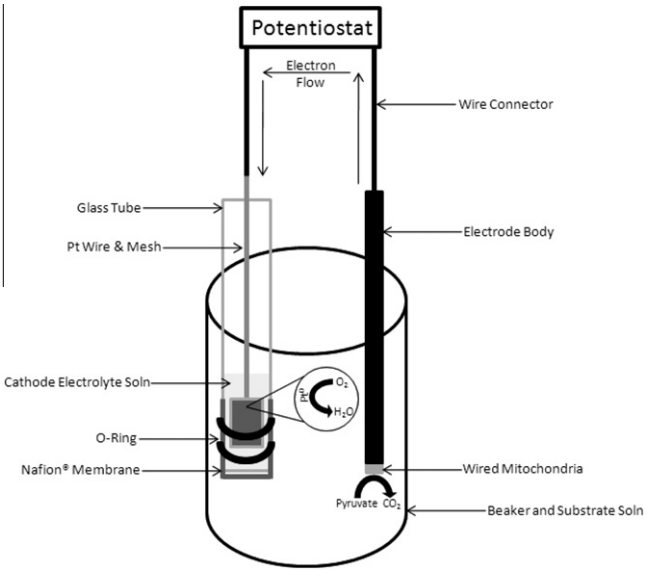


Figure 1. Schematic of the electrochemical setup for measuring mitochondrial function.

in the living cell.[20,26] The fabrication method for forming mitochondrial electrodes allows access to the cytochrome c which resides in the intermembrane space.[20]

We have found in previous studies that this electrochemical mitochondrial assay system is sensitive towards many potent mitochondrial inhibitors, including: rotenone, oligomycin, DIM, and cyanide.[20,21,26,27] These inhibitors directly inhibit the ETC complexes and results in metabolic changes in the function of mitochondria. In this study, we utilize our mitochondrial modified electrodes to investigate specific mitochondrial CAIs to determine the overall metabolic effects of the CAIs on the mitochondria when the mitochondria are metabolizing different metabolic substrates (i.e. pyruvate, fatty acids, etc.). CA is not an ETC complex, but it affects metabolism and therefore should affect the long term ETC electron throughput. With this electrochemical method, we attempted to shed light on which metabolic pathways are being affected and to what degree by the inhibition of the mitochondrial hCA VA and VB. In the first set of experiments, the effect of the inhibitors was determined using a pyruvate solution as the metabolic substrate solution. The overall metabolic energy conversion (power) was measured electrochemically from polarization measurements. Metabolic energy conversion (power) is a function of electron flow (current) and electrochemical potential (voltage). Representative comparisons of pyruvate metabolism in the presence and the absence of inhibitor compound **2** are shown in Figure 2. The data is shown in terms of polarization curves showing maximum open circuit potential (potential at 0A) and maximum current density (current at 0 V) and in terms of power curves which utilize the same data, but plot power versus current to determine the maximum power or metabolic energy conversion of the mitochondria. The tabulated open circuit potentials, maximum current density, and maximum power densities before and after introduction of the inhibitor for pyruvate as a metabolic substrate are shown in Table 2. All of these CAIs had very similar attenuation effects on the mitochondrial energy conversion rate (power) and open circuit potential when pyruvate was used as the sole energy source indicating that the CA associated with pyruvate carboxylase was being affected strongly. There is minimal effect on maximum current density, which was expected, because these inhibitors are not ETC complex inhibitors and CA is significantly farther up the metabolic pathway.

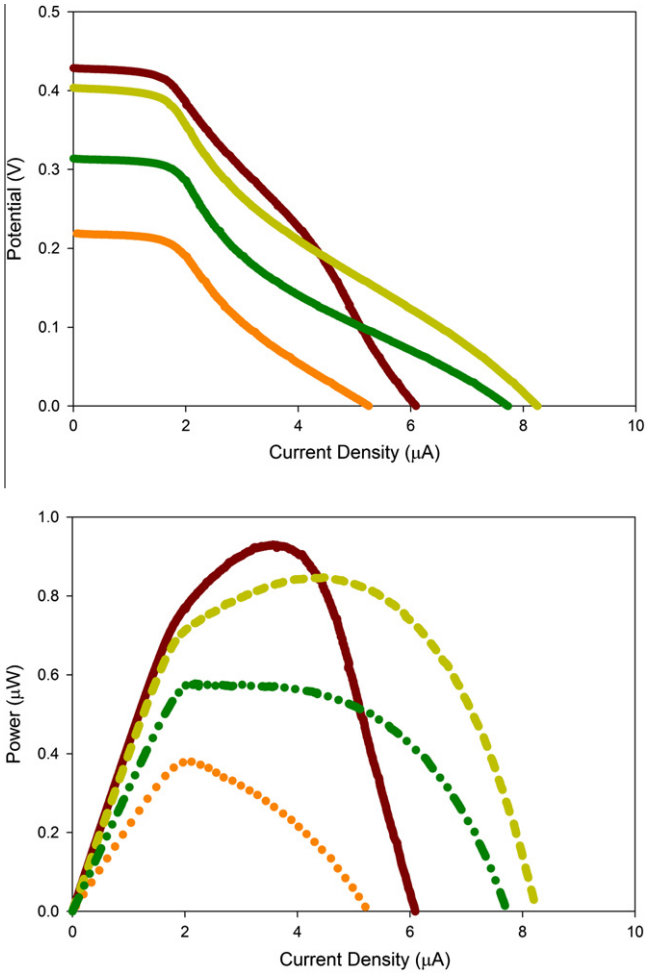


Figure 2. Representative polarization curves (top) and power curves (bottom) for carbonic anhydrase inhibitor compound **2** on the metabolism of pyruvate (before inhibitor shown in brown and after inhibitor shown in orange) and the metabolism of succindate (before inhibitor shown in pale green and after inhibitor shown in dark green).

Table 2
Table of data for electrochemical experiments run in 100 mM pyruvate solution before and after the introduction of 1 μM of each of the inhibitors

Inhibitor w/ pyruvate substrate	Open circuit potential (mV)	Maximum current (μA)	Maximum power (μW)
1 Before inhibitor	379 ± 68	7.2 ± 1.5	1.1 ± 0.4
1 After inhibitor	257 ± 19	6.8 ± 1.4	0.45 ± 0.08
2 Before inhibitor	437 ± 29	7.4 ± 1.5	1.1 ± 0.5
2 After inhibitor	299 ± 90	7.4 ± 2.4	0.42 ± 0.06
3 Before inhibitor	347 ± 25	8.3 ± 0.1	0.85 ± 0.08
3 After inhibitor	259 ± 35	9.0 ± 0.9	0.52 ± 0.12
4 Before inhibitor	441 ± 98	7.0 ± 1.6	1.1 ± 0.2
4 After inhibitor	309 ± 10	7.0 ± 0.2	0.53 ± 0.04
5 Before inhibitor	345 ± 7	9.7 ± 3.4	1.0 ± 0.3
5 After inhibitor	244 ± 11	6.3 ± 2.8	0.44 ± 0.11

In order to study the effect of metabolic substrate on mitochondrial metabolic inhibition by CAIs, we varied the metabolic substrate from pyruvate to acetate. Table 3 shows tabulated open circuit potentials, maximum current density, and maximum power density for acetate as a metabolic substrate. When acetate was used as the sole source of energy for the mitochondria, the CAIs had less of an effect on maximum energy conversion power on average for all of the inhibitors. This indicates that mitochondrial metabolism is affected less by acetate than pyruvate, suggesting in organisms, these types of compounds would likely promote weight loss due to the flux of metabolism being shifted towards fatty acid oxidation.

One of the more interesting results was observed when succinate was used as the sole metabolic substrate. Table 4 shows tabulated open circuit potentials, maximum current density, and maximum power densities for succinate as a metabolic substrate. These experiments showed that succinate metabolism (i.e. the energy conversion derived from the power measured electrochemi-

Table 3
Table of data for experiments run in acetate solution before and after the introduction of 1 μM of each of the inhibitors

Inhibitor w/acetate	Open circuit potential (mV)	Maximum current (μA)	Maximum power (μW)
1 Before inhibitor	260 ± 42	8.1 ± 2.9	0.56 ± 0.14
1 After inhibitor	218 ± 11	7.9 ± 0.4	0.37 ± 0.03
2 Before inhibitor	413 ± 19	9.4 ± 1.1	0.91 ± 0.31
2 After inhibitor	297 ± 9	9.1 ± 0.4	0.61 ± 0.02
3 Before inhibitor	293 ± 32	6.3 ± 1.0	0.51 ± 0.08
3 After inhibitor	224 ± 3	5.4 ± 1.0	0.35 ± 0.04
4 Before inhibitor	354 ± 22	8.4 ± 1.1	0.68 ± 0.06
4 After inhibitor	274 ± 4	7.7 ± 1.5	0.59 ± 0.06
5 Before inhibitor	336 ± 3	11.8 ± 2.5	1.1 ± 0.2
5 After inhibitor	278 ± 4	9.8 ± 2.0	0.62 ± 0.93

Table 4
Table of data for experiments run in succinate solution before and after the introduction of 1 μM of each of the inhibitors

Inhibitor w/succinate	Open circuit potential (mV)	Maximum current (μA)	Maximum power (μW)
1 Before inhibitor	254 ± 29	11.9 ± 3.6	0.80 ± 0.32
1 After inhibitor	293 ± 12	12.6 ± 4.4	0.82 ± 0.26
2 Before inhibitor	405 ± 3	8.3 ± 1.1	0.84 ± 0.06
2 After inhibitor	316 ± 4	7.7 ± 0.8	0.58 ± 0.01
3 Before inhibitor	323 ± 11	9.6 ± 0.7	0.67 ± 0.03
3 After inhibitor	276 ± 10	9.2 ± 1.2	0.51 ± 0.03
4 Before inhibitor	365 ± 6	12.7 ± 0.8	1.0 ± 0.1
4 After inhibitor	329 ± 5	12.0 ± 1.4	0.79 ± 0.09
5 Before inhibitor	366 ± 5	11.6 ± 2.7	1.1 ± 0.2
5 After inhibitor	304 ± 8	9.7 ± 2.3	0.66 ± 0.11

cally) was affected the least across the board for all of the inhibitors. This is consistent with theory, because it has the furthest carbon dioxide producing or utilizing pathway step of the substrates tested. Consequently, metabolism is bottlenecked much further downstream in the metabolic pathway than the other substrates and that there are more electron producing steps before the bottleneck. Most interesting were inhibitor **1** and inhibitor **5**. Inhibitor **1** seems to only have a significant effect on pyruvate and acetate metabolism, but has minimal effect on succinate metabolism. On the other hand, inhibitor **5** appears to be the strongest inhibitor of metabolism as shown by a 55.6% attenuation in pyruvate metabolism, a 44.1% attenuation in acetate metabolism, and a 41.1% attenuation in succinate metabolism.

4. Conclusions

Overall, the effectiveness of these compounds can be examined by using the wired mitochondrial electrochemical assay in order to show the different effects of each inhibitor on each substrate's metabolic pathway. This information can be very useful during drug design when a particular target is being sought. While it is not able to yield specific data about individual enzyme kinetics like an enzyme assay, it can be used in addition to enzyme assay data to confirm that the indicated pathway is being affected without collateral pathways being affected. This becomes important when drug side effects are being considered, because every other pathway that is affected by the drug can potentially cause unwanted or detrimental side effects. Ideally, for an antiobesity drug, the flux of the pyruvate carboxylase pathway should be attenuated, but all other pathways should remain allowing for more fatty acid oxidation and less glucogenesis or lipogenesis. Therefore, inhibitor **1** is an interesting drug candidate, because it affects pyruvate oxidation to a far greater extent than acetate oxidation, whereas inhibitor **5** affects both pyruvate and acetate oxidation in a similar way.

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