Response to Perspectives on the Classical Enzyme Carbonic Anhydrase and the Search for Inhibitors

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In a recent paper by Jonsson and Liljas (1), some inhibition data from the supporting information of several papers from the Supuran group were considered where carbonic anhydrase (CA, EC 4.2.1.1) inhibitors (CAIs) were reported (2–10). The concentrations of the enzyme and substrate concentrations used in some experiments were questioned, and the authors assumed that the measurements were performed incorrectly (1). For example, Jonsson and Liljas state that ''inhibition curves [from the supporting information in a few of our papers] do not show normal inhibition.'' This and other statements from this paper (1) are fully inconsistent and are based on conceptual errors, which will be detailed here.

1) ''An inhibitor of an enzyme should at some concentration abolish the enzyme activity almost completely''.'' Jonsson and Liljas (1) based their analysis on a ''theoretical curve'' and assumed that at increasing concentrations of an effective inhibitor, the enzyme activity should reach zero. This is conceptually erroneous because the reaction between and enzyme (E) and an inhibitor (I) is an equilibrium process (E + I ↔ EI) and even for very tight binding inhibitors, there will always be free enzyme available to perform catalysis. This is particularly relevant for the CAs, enzymes that are highly efficient at catalyzing the hydration of CO_2 to form bicarbonate and protons (11). In most of our papers we use two different CA assays: the first based on a stopped-flow CO_2 hydrase assay,

reported in 1971 by Khalifah (12) and validated by others as a rapid method for determining the enzyme kinetics and inhibitory constants of various classes of compounds against different CAs (13,14); and the second is the esterase assay, which involves 4-nitrophenyl acetate as a substrate and is based on the method developed by Pocker (15). By using both assays and a variety of human and non-human CA isoforms, an activity of zero, even in the presence of high concentrations of inhibitor, has never been reported in the literature.

2) ''A low-molecular-weight inhibitor cannot inhibit more than one enzyme molecule.'' We agree with this statement and we have never claimed otherwise.

3) ''The inhibition by a strongly binding inhibitor has to be measured at a correspondingly low enzyme concentration.'' The criticism from Jonsson and Liljas (1) is based on their statement that the enzyme and substrate concentrations used in some of our experiments were not mentioned in the paper or were exceedingly high. We should note that many of the stopped flow assay papers cited above (13,14) which are not from Supuran's group but from several well-established laboratories, provided exactly the same level of information found in our papers—the range of CO_2 concentrations at which the experiments were performed was reported, without detailing enzyme concentrations (13,14). However, in many of our papers the enzyme concentrations was reported either for the CO_2 hydrase or esterase assays (16–18) and they always ranged between 3.5 and 14 nM. The analysis performed by Jonsson and Liljas (1) is based on supplementary information that was reported in six of our papers (2–7). Two of the other papers (8,9) reported CA inhibitory data as well which was obtained in a different laboratory. They also erroneously cited the wrong paper (10) for the development of SLC-0111, which is a compound that is currently in Phase I/II clinical trials as an antitumor agent. The presumed lack of accuracy could result from typographical errors for enzyme concentrations in a single paper's supplementary information (3); i.e., a concentration of 107 M was reported, which is, in actuality, the enzyme concentration of the stock solution and not the concentration at which the assay was performed. As mentioned above, in most of our experiments, we work at enzyme concentrations ranging from 3.5 and 14 nM and sometimes even lower when exceedingly strong inhibitors are analyzed. Jonsson and Liljas (1) also questioned the enzyme inhibition curves from the supplementary information of another paper (4). For those figures, the uncatalyzed reaction was not subtracted in the curves plotted in the supplementary figures. However, the background activity from the uncatalyzed reaction is always subtracted when IC_{50} values are obtained by using the data analysis software for the stopped flow method. Errata regarding these errors have been submitted to both journals to correct the supplementary information. Furthermore, inhibition constants (Kis) of more than 30 CAIs obtained by stopped flow kinetic measurements at the University of Florence have been compared to the dissociation constants (Kds) measured by native mass spectrometry (MS) in a different laboratory (19,20). Data obtained by both methods for six well-known sulfonamide CAIs with hCA I and II are shown in Table 1. For inhibitors that bind at the site of the substrate, Kd and Ki values were found to be equivalent and consequently, the results obtained by both native MS and the stopped flow kinetic method are in excellent agreement. For example, the measured Kd values of all nanomolar inhibitors were within an average of 30% of the Ki values. More recently, 30 Kd and Ki values were obtained for 15 perfluoroalkyl substances interacting with either carboxylate, sulfate, or sulfonamide zinc binding groups of hCA I and hCA II by use of the stopped flow and native MS methods (20), all of which also resulted in excellent agreement; i.e., the Kd values were within an average of 37% of the Ki

values. These results provide additional support that Ki values can be accurately obtained using the stopped flow approach for identifying CAIs for drug development applications.

Table 1. Measured dissociation and inhibition constants (μM) of six sulfonamides to hCA I and hCA II obtained using native mass spectrometry and a stopped flow kinetic inhibition assay respectively.

	hCA I		hCA II	
Inhibitor	Native MS	Stopped Flow	Native MS	Stopped Flow
Ethoxzolamide	0.014 ± 0.002 [33]	0.025[a] [36], 0.009[b] [37]	0.010 ± 0.001 [33]	0.008[a] [36]
Brinzolamide	1.06 ± 0.05 [33]	0.73 ± 0.04[a] [38]	0.005 ± 0.001 [33]	0.003[a] [38]
Furosemide	0.055 ± 0.005 [33]	0.062[a] [33]	0.098 ± 0.009 [33]	0.065[a] [38]
Dichlorophenamide	1.3 ± 0.1 [33]	1.2[a] [39]	0.027 ± 0.002 [33]	0.038[a] [39]
Indapamide	9.2 ± 0.3 [33]	51.9[a] [40]	3.22 ± 0.20 [33]	2.52[a] [40]
Acetazolamide	0.24 ± 0.02 [33]	0.25[a] [36]	0.015 ± 0.001 [33]	0.012[a] [36]

[a]U. Florence stopped flow kinetic inhibition constant measurements (μM) [23-32]. [b]U. Florida buffer indicator kinetic method measurements [37].

4) ''In the calculation of the Ki from an estimated IC_{50}, the numeric value of the Ki must be smaller than the IC_{50}.'' We also agree with this statement of Jonsson and Liljas (1). In fact, in our papers, either the Ki or the IC_{50} was reported. As mentioned above, there were two papers in which enzyme assays were done in other laboratories (8,9), and these papers reported both Ki and IC_{50} values as well. Our co-authorship on these papers involved organic synthesis and not the enzyme assays, which were the responsibility of other authors.

5) ''In a scientific work, the primary data should be provided in the main manuscript or the supplement so that the experiments can be reproduced and the calculations can be checked.'' We have measured the inhibition constants for more than 10,000 different compounds belonging to a range of diverse classes, such as sulfonamides, sulfamates, sulfamides, coumarins, sulfocoumarins, homosulfocoumarins, homocoumarins, thiocoumarins, phosphonamidates, dithiocarbamates, monothiocarbamates, xanthates, trithiocarbonates, selenols, carboxylates hydroxamates, benzoxaboroles, carbamates, and polyamines (review in 26). Jonsson and Liljas criticize that ''the data are often provided in tables, in which important information and the original measurements are lacking.'' In all medicinal chemistry journals, data are provided in tables, and the primary inhibition data for compound screening is not normally included. As mentioned above, in the experimental part of our papers, detailed information on the assays are provided. Furthermore, extensive kinetic, crystallographic, mass spectrometry, NMR, and synthetic efforts led to a thorough understanding of several innovative CA inhibition mechanisms (26,27), including the anchoring of CAIs to the zinc coordinated water molecule, occlusion of the active site entrance, and even binding outside the active site cavity (26). Any additional primary data that is required can be readily obtained upon request from the corresponding author. The last statement from Jonsson and Liljas (1) refers to the sulfonamide drug candidate SLC-0111 that was discovered in our laboratories and is now in Phase Ib/II clinical trials for the treatment of advanced metastatic solid tumors (28). Although there are no drug design or oncology studies published by Jonsson or Liljas, and although there is no new information or data in their paper (1) regarding SLC-0111, the two authors state that ''it is doubtful whether the compound has the required Ki to be advanced into clinical trials'' and cite a reference which has no connection with the compound. SLC-0111, the lead clinical CA IX/CA

XII inhibitor, was identified by the Supuran group through a program in which several thousands of sulfonamides were prepared and assayed as CAIs toward several physiologically relevant human isoforms (29). It belongs to a family of ureidobenzene sulfonamides, which were assessed for ''druggability'' criteria, including ADME (Absorption, Distribution, Metabolism, Excretion) analysis, from which SLC-0111 was selected for further in vitro and in vivo analysis in appropriate cancer models (30). For reasons of space we are unable to cite the large number of drug design in laboratories all over the world, which used SLC-0111 as a lead compound. The evaluation of CA IX/CA XII inhibitors as anti-cancer compounds takes a different path from the development of other cytotoxic anti-cancer drugs. This is because the targets, CA IX/CA XII are only expressed within the hypoxic niches of solid tumors, and may represent a minor portion of the total tumor cell population, which significantly contribute to resistance to chemo-, radiation-, and immuno- therapies (31). The extensive pre-clinical models carried out by several independent groups with several CA IX/XII inhibitors, including SLC-0111, have demonstrated that the use of such inhibitors in combination with chemotherapy agents, immunotherapy and radiotherapy (31,32) is highly important and desirable for sustained therapeutic response. The extensive studies reported in these and other recent papers, utilizing multiple in depth in vivo models, provided solid positive pre-clinical data to warrant the initiation of Phase 1 clinical trials in 2014, of which a Phase 1 safety trial with SLC-0111 (as a monotherapeutic agent) has been completed (28) and a Phase 1b trial is currently underway to evaluate SLC-0111 in combination with gemcitabine in metastatic pancreatic cancer patients whose tumors are CA IX positive (ClinicalTrials.gov Identifier: NCT03450018). In conclusion, through rigorous pre-clinical investigations involving crystallographic, spectroscopic, and synthetic efforts, and the screening of tens of thousands of potential inhibitors of CAs by use of a functional, stopped-flow kinetic inhibition assay, lead compounds for CA IX and XII, including the sulfonamide CA inhibitor SLC-0111, have been identified, validated, and developed. The results from the stopped flow kinetic enzyme inhibition assay agree very well with orthogonal native mass spectrometry measurements for directly measuring protein-ligand interactions and kinetic inhibition measurements from other labs that do not involve a stopped flow approach. Overall, the stopped flow kinetic enzyme inhibition method has been used to screen tens of thousands of potential CAIs accurately, enough for medicinal chemistry and pre-clinical pharmacology applications. Given the rich and abundant literature establishing and validating specific CA IX and CA XII inhibitors, such as in pre-clinical models of cancer and in clinical trials, these isoenzymes are promising cancer targets for therapy in advanced, hypoxic tumors. In the future, the use of selective CA IX/XII inhibitors, such as SLC-0111, should be advantageous in combination with chemo-, radiation-, and immuno-therapies to eliminate resistant cancer cell populations and to maximize the suppression of tumor growth and metastasis. A more detailed account of these issues, in the context of targeting the tumor microenvironment will be published elsewhere.

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