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Assessment of Free Drug Concentration in Cyclodextrin Formulations Is Essential to Determine Drug Potency in Functional *In Vitro* Assays

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ABSTRACT

Cyclodextrins (CD) have the ability to form inclusion complexes with drugs and can be used as excipients to enhance solubility of poorly soluble drugs. To make accurate estimations of the potency of the drug, knowledge of the free drug concentration is important. The aim of this study was to evaluate the applicability of calculated free drug concentrations toward response measurements in a transient receptor potential vanilloid receptor-1 cell-based *in vitro* assay. This included accounting for potential competitive CD binding of 2 transient receptor potential vanilloid receptor-1 active entities: 1 antagonist, and 1 agonist (capsaicin). Solubility of the CD-drug complexes was measured, and the ligand to substrate affinity in CD formulations was determined according to the phase-solubility technique. The total concentration of antagonist, agonist, CD, and the binding constants between ligands and CD were used to calculate the free concentration of CD ligands. For capsaicin and 2 of the 3 investigated model drugs, the calculated free drug concentration was consistent with the experimental *in vitro* data while it was overestimated for one of the compounds. In conclusion, the suggested approach can be used to calculate free drug concentration and competitive binding in CD formulations for the application of cell-based drug functionality assays.

Introduction

The pharmaceutical industry has during the last decades struggled to maintain the output of new pharmaceutical entities of small molecule origin due to a continuous high attrition rate of candidate drugs.[1] One contributing factor to this is an increased number of compounds with low aqueous solubility. This is partly a consequence of the pharmacological targets of interest, but also the increased application of combinatorial chemistry tools.[2] Low solubility introduces both practical and physiological problems for drug development.[3] One approach to improve the solubility of a poorly soluble compound can be by the use of a solubility enhancer such as cyclodextrins (CD).[4-6] CDs are oligosaccharides with a hydrophobic central cavity and a hydrophilic external surface.[7] Several CDs are approved as an excipient for use in man by the U.S. Food and Drug Administration and other regulatory agencies.[4] If a molecule's dipole moment and size correspond to the central cavity of the CD, an inclusion complex can be formed which increases the total solubility of the binding molecule.[6] The most common technique for determination of binding constants between drugs and CDs (1:1 complexes) is the use of phase diagrams, a technique that was introduced by Higuchi and Connors in 1965.[8] Phase diagrams have since then also been used to assess 1:2 and 1:3 complex formation between the drug and the CD.[9] Competitive binding to CD may occur *in vitro* if 2 compounds have a CD affinity at the same magnitude.[10] In the case where 2 compounds with competitive properties are present, the free concentration of each compound can be calculated if (1) the total concentrations and (2) their respective affinities for the CD are known. One way of calculating the free concentrations of compounds that competitively bind to CD has been presented by Funasaki and et al.[10] In addition to these *in vitro* physical-chemical

Abbreviations used:: CD, cyclodextrin; CD_{tot}, total concentration of substrate; CHO, Chinese hamster ovary cells; EC_{50}, half maximal effective concentration; FLIPR, fluorometric imaging plate readers; $f_{u,cal}$, calculated free fraction; IC_{50}, half maximal inhibitory concentration; ICVR1, TRPV1 antagonist/TRPV1 inhibitor, investigational compound for vanilloid receptor 1; k, slope of the phase-solubility diagram; $K_{1:1}$, binding constant; L_t, total concentration of solubilized ligand; TRPV1, transient receptor potential vanilloid receptor-1.

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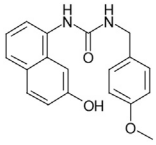
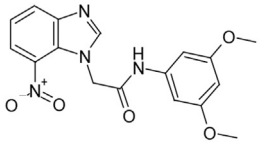
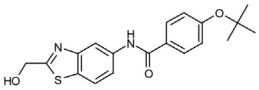
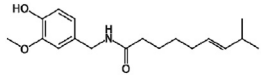
Table 1
Chemical Structures and Physicochemical Properties of the Investigational Compounds for Vanilloid Receptor 1 (ICVR1 Compounds or TRPV1 Antagonists/Inhibitors) and Capsaicin (TRPV1 Agonist/Activator)

Compound	AZ11760788	AZ12048189	AZ12099548	Capsaicin
Structure				
Molecular weight (g/mol)	322	356	356	305
Log $D_{pH7.4}$[a]	3.29	2.40	3.36	3.22
pK_a	9.2 (acid)	2.2 (base)	1.6 (base)	9.8 (acid)
S, pH = 7.3 (μM)	8.0	8.3	5.0	107

Solubility (S at pH 7.3) and pKa values were determined experimentally, while log $D_{pH7.4}$ values were estimated.
[a] Estimated with ACD/log D v8.0 and v12.0 (Advanced Chemistry Development, Inc., Toronto, ON).

properties of [CD + drug] equilibria, administration of a CD formulated compound to a living cell culture is further complicated as several additional equilibria, for instance membrane permeability, must be taken into account.[11,12]

The transient receptor potential vanilloid receptor-1 (TRPV1) is a highly Ca^{2+} permeable, non-selective cation channel that responds to a variety of selective activators including compounds such as capsaicin (pungent ingredient in hot chili peppers).[13,14] The binding site of capsaicin is located on the intracellular side of the channel, close to the membrane, and activation of TRPV1 evokes a sensation of burning pain.[15] As a consequence, TRPV1 is of interest as a therapeutic target to manage pain.[16,17] In the search for new potent pharmaceutical compounds, large efforts have been done in the development of high-throughput screening techniques.[18-20] Fluorometric Imaging Plate Readers (FLIPR) in combination with cells overexpressing TRPV1 is a common high throughput *in vitro* method used to identify TRPV1 active compounds.[21] Due to the nature of this functional assay—and the nature of the binding site on the TRPV1 target—the bioactive compounds identified using this assay are typically difficult to solubilize in balanced saline solutions. Therefore, solubilization of pharmacologically active compounds is necessary. If compounds are formulated with a solubility enhancer such as CD, an estimation of the free drug concentration by calculation is necessary to determine the potency of the drug.

The aim of this study was to test TRPV1 active compounds in CD formulations on a functional *in vitro* FLIPR assay, in order to compare calculated estimations of free drug concentrations with measured data from an experimental test system. Calculations were performed using a derived expression for competitive binding to a CD in the presence of 2 CD-binding molecules. This investigation was critically important in order to design future *in vivo* evaluations and to deduce pharmacological conclusions on these results.

Materials and Methods

Chemicals

Phosphoric acid 85% and Tris-(hydroxymethyl)-aminomethane (TRIS) and methanol 99.7% were purchased from Merck (Damstadt, Germany). Acetonitrile Ultra Gradient HPLC Grade (high-pressure liquid chromatography) was purchased from J.T. Baker (Deventer, Holland). Sodium hydroxide was purchased from KEBO-LAB (Spånga, Sweden). Capsaicin was purchased from Sigma (St. Louis, MO) and hydroxypropyl-β-cyclodextrin (Kleptose HPBJ P15, lot no: E0004) was purchased from Roquette (Lestrem, France).

Three TRPV1 antagonist (ICVR1) compounds synthesized at AstraZeneca (AZ11760788, AZ12048189, and AZ12099548) as well as capsaicin (TRPV1 agonist) were used in this study to evaluate the calculations of the free drug concentration in CD formulations when determining pharmacological effect in *in vitro* cell systems (Table 1).

pKa Determination of TRPV1 Antagonist

A GLpKa instrument equipped with a D-PAS spectrophotometric detection unit from Sirius Analytical Instruments Ltd. (East Sussex, UK) was used for the determination of the investigated ICVR1s pKa values. A proper amount of the compound was weighted and dissolved in dimetylsulfoxid (DMSO) to a final concentration of 10 mM. A total of 15 μL of the DMSO stock solution was first buffered with 250 μL phosphate buffer pH 7.0 and then titrated at 25°C from low to high pH (pH 1.8-12.2) in a 0.15 M KCl aqueous solution. Final pKa values were calculated using the Refinement Pro software (Sirius Analytical Ltd., East Sussex, UK).

Buffer Solubility Measurements

The buffer solubility was measured in phosphate/TRIS buffer pH 7.3. Excess of each of the investigated compounds were added to the buffer to create a saturated system and the suspensions were shaken at 25°C for 24 h. After visual examination that solid material still remained in the vial, the samples were centrifuged in order to remove solid particles from the solution. The supernatants were assayed on a HPLC Agilent HP1100 Series gradient system equipped with an injector, 2 high-pressure pumps, and a diode array detector or a ultraviolet detector (Agilent Corporation, Santa Clara, CA). The concentration of the drugs was calculated using the peak areas by the software (Chemstation v10.01). The mobile phase consisted of a mixture of acetonitrile and phosphate buffer pH 3.0, I = 0.01, solvent A 100/900 (vol/vol), and solvent B 900/100 (vol/vol). The flow rate was 0.5 mL/min. The column was a Waters Symmetry C8 column, 2.1 × 50 mm packed with 3.5 μm particles (Waters Corporation, Milford, MA). An aliquot of 30 μL was injected and detection was performed at 230 and 250 nm. The gradient ramp was 0-7 min, 0%-100% B; 7-10 min, 100% B; and 10-12 min, 100%-0% B.