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# Interaction of protein phosphatase inhibitors with membrane lipids assessed by surface plasmon resonance based binding technique

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ABSTRACT

The interaction of okadaic acid (OA), tautomycin (TM), microcystin-LR (MC-LR), cantharidin (CA), epigallocatechin-gallate (EGCG) and cyclosporin A (CsA), inhibitors of protein phosphatases, with liposome covered surfaces prepared from the lipid extracts of bovine brain, heart and liver was investigated by surface plasmon resonance (SPR) based binding technique. The SPR sensorgrams indicated reversible association or partial intercalation of the inhibitors with liposomes at 20 °C or 37 °C, respectively. Distinct lipid composition specificities were reflected in different saturation values of inhibitor binding in a decreasing order of liver > heart >> brain lipids. Assaying the effect of OA, TM, MC-LR, CA and EGCG on the activity of protein phosphatases in neuroblastoma B50, cardiomyoblast H9C2 and hepatocarcinoma HepG2 cells implied that the cell type specific association of phosphatase inhibitors with membrane lipids may influence their inhibitory potencies exerted on intact cells.



## 1. Introduction

Reversible phosphorylation of proteins on serine (Ser), threonine (Thr) and tyrosine (Tyr) residues is a major regulatory device in many cellular processes (Cohen, 2002). More than 90% of protein phosphorylation occurs at Ser/Thr residues, therefore, identification of the interconverting enzymes, the Ser/Thr specific protein kinases and phosphatases, involved in specific cellular events, have received widespread attention for the past few decades. Initially, the protein kinases were assumed as major targets of regulatory interventions, while the protein phosphatases were thought of as the necessary "housekeeping" enzymes to ensure the reversibility of phosphorylation (Brautigan, 2013). It has become clear, however, that the protein phosphatases are also regulated by a wide variety of mechanisms (Bollen et al., 2010; Hartshorne et al., 2004) and they are important mediators of the strength and duration of the cellular signals (Heinrich et al., 2002). It is also well established that three types of Ser/Thr specific phosphatases, protein phosphatase-1 (PP1), -2A (PP2A) and -2B (PP2B) are responsible for the vast majority of dephosphorylation of phospho-Ser/Thr residues in proteins (Mansuy and Shenolikar, 2006).

The discovery of specific, membrane permeable inhibitors of PP1, PP2A and PP2B, and their application to cells permitted to assess the physiological functions of these enzymes (Honkanen and Golden, 2002). Okadaic acid (OA) was the first PP1 and PP2A inhibitory toxin identified (Takai et al., 1987), followed by calyculin-A (CLA) (Ishihara et al., 1989) and tautomycin (TM) (MacKintosh and Klumpp, 1990). OA, CLA and TM are ineffective on PP2B, or inhibit its activity only at much higher concentrations than they do PP1 or PP2A. In contrast, cyclosporin A (CsA) forming a complex with cyclophilin in cells specifically inhibits PP2B, but does not influence directly PP1 or PP2A (Ke and Huai, 2004; Rusnak and Mertz, 2000). Later, microcystin-LR (MC-LR), a hepatotoxic cyclic heptapeptide produced by cyanobacteria (MacKintosh et al., 1990) as well as cantharidin (CA) and its derivatives were also identified as inhibitors of PP1 and PP2A (Li and Casida, 1992). The latter compounds also exerted cytotoxic effects not only on hepatic tissue but other cells, too (Erdődi et al., 1995). Recently, polyphenolic compounds such as penta-*O*-galloyl-β-D-glucose (PGG) as well as epigallocatechin-gallate (EGCG) and its derivatives were shown to inhibit PP1 with preference compared to PP2A (Kiss et al., 2013). The structural backgrounds for the protein phosphatase–inhibitor complexes have been uncovered in several cases and these data may initiate the design of drugs for type specific regulation of these enzymes (Zhang et al., 2013).

While the inhibitory potency and selectivity of the phosphatase inhibitory molecules to the different types of enzymes are quite well characterized *in vitro*, their action, effective concentrations

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and targeted enzymes in the cells are less known. Favre et al. (Favre et al., 1997) characterized the effects of OA, CLA and TM on the phosphatase activity of different cell lines and concluded that OA had relative specificity toward inhibition of PP2A, while TM preferentially blocked PP1 in cells, whereas CLA suppressed the activity of both enzymes. In addition, the extent of inhibition exerted by these molecules was dependent upon the duration of their incubation with cells.

It is assumed that the permeation of the inhibitors through the cell membrane could be an important factor in the development of the inhibitory effect, and these processes may be dependent upon the cell types with cell membranes of different lipid constituents. The present study was undertaken to characterize the association-dissociation features of distinct phosphatase inhibitory molecules with lipid vesicles derived from different tissues by surface plasmon resonance (SPR) based binding technique. The liposomes prepared from the lipid mixtures representing the compositions characteristic for brain, heart and liver tissues were immobilized on L1 sensor chip and binding of inhibitors were recorded as sensorgrams. It is seen that the association of inhibitors with liposomes differs depending on the temperature and the tissue specific composition and ratio of lipids implying possible differences in the membrane permeation of these molecules in various tissues and cells. Consistent with these findings, in phosphatase activity assays the inhibitory potencies of these molecules proved to be different in neuroblastoma B50, cardiomyoblast H9C2 and hepatocarcinoma HepG2 cell lines.

## 2. Materials and methods

### 2.1. Materials

Chemicals and vendors were as follows: [$\gamma$-$^{32}$P]ATP was from Hungarian Isotope Institute (Budapest, Hungary); L-glutamine, DMEM and fetal bovine serum were from PAA Laboratories (Pasching, Austria); MC-LR isolated and purified as described (Máthé et al., 2009) was a gift from C. Máthé (Department of Botany, University of Debrecen); EGCG, OA, TM, BSA and Chaps were from Sigma–Aldrich (St. Louis, MO, USA); CA was from LC Services (Woburn, MA, USA); Complete Mini Protease Inhibitor Cocktail was from Roche (Penzberg, Germany); Sensor Chip L1 was from Biacore AB (Uppsala, Sweden); Bovine brain, heart and liver total lipid extracts were from Avanti Polar Lipids (Alabaster, Alabama, USA). All other chemicals used were of the highest purity commercially available.

### 2.2. Liposome preparation

The composition of brain, heart and liver total lipid extracts are shown in Table 1 as given by the description of the commercial source, Avanti Polar Lipids. Lipids were prepared in the same way, regardless of their compositions using the methodology described previously (Abdiche and Myszka, 2004). Briefly, lipid extract powders were hydrated at room temperature in running buffer (50 mM Hepes, 150 mM NaCl, pH 7.4) used in the interaction analysis. Lipid suspensions (~3 mg/ml) were subjected to four cycles of freezing (−80 °C), thawing (20 °C), and vortexing (5 s) to ensure thorough agitation prior to extrusion through a polycarbonate filter of defined pore diameter (100 nm) using an Avanti Mini-Extruder kit. This involved sandwiching a membrane between two syringes, loading the crude lipid suspension into one syringe, and passing it many times (a minimum of 15) through the membrane. The extruded product, containing uniformly sized micelles was always unloaded from the opposing syringe to avoid contamination present in the original sample. The prepared liposomes were diluted in running buffer to 1 mg/ml.

**Table 1**
The composition of the total lipid extracts of brain, heart and liver.

| Component | (%/wt.) |
|---|---|
| Brain total lipid extract | |
| Phosphatidylethanolamine | 16.7 |
| Phosphatidylserine | 10.6 |
| Phosphatidylcholine | 9.6 |
| Phosphatidic acid | 2.8 |
| Phosphatidylinositol | 1.6 |
| Other | 58.7 |
| Heart total lipid extract | |
| Phosphatidylethanolamine | 6.8 |
| Phosphatidylinositol | 2.5 |
| Cardiolipin | 2.3 |
| Phosphatidylcholine | 5.4 |
| Phosphatidic acid | 1.1 |
| Neutral lipid | 49.8 |
| Other | 32.1 |
| Liver total lipid extract | |
| Cholesterol | 7.0 |
| Phosphatidylethanolamine | 22.0 |
| Phosphatidylinositol | 8.0 |
| Phosphatidylcholine | 42.0 |
| Lysophosphatidylinositol | 1.0 |
| Others, including neutral lipids | 20.0 |

### 2.3. Capture of liposomes on the L1 sensor chip

The L1 sensor chip surfaces were washed with three consecutive one minute pulses of 20 mM Chaps at a flow rate of 100 μl/min, followed by the rinsing routine Extraclean. Liposomes were captured across isolated flow cells around saturation to approximately the same immobilization level (~7500 RU) at a flow rate of 2 μl/min. The reference (lipid free) and the three lipid surfaces were blocked with 0.1 mg/ml BSA at a flow rate of 5 μl/min for 15 min. BSA binds in a high amount to the L1 chip surface composed of dextran matrix modified with lipophilic moiety (Cho et al., 2004; Erb et al., 2000). The flow rate then was switched to 100 μl/min and the surfaces were washed with running buffer for three minutes.

### 2.4. SPR measurements

The interactions were investigated at 20 and 37 °C using Biacore 3000 instrument equipped with an L1 sensor chip. The protein phosphatase inhibitors were diluted in 10 mM Hepes (pH 7.4) plus 150 mM NaCl to give a concentration series. The Kinject injection type was used and 120 s durations were chosen for both the association and dissociation phases. Inhibitor samples were dispensed into single-used snap-capped vials and injected across the three different lipid and the lipid-free (control) surfaces in a single step. In case of all inhibitors the injections were repeated with increasing sample concentration in successions. The application of these successive injections was possible since the inhibitors dissociated completely from the surfaces during the dissociation phase. After each binding series, the sensor surfaces were regenerated to the original matrix by injecting 4:6 v/v% isopropanol/50 mM NaOH at a flow rate of 100 μl/min followed by washing with 20 mM Chaps. The sensor surfaces were recoated with fresh liposome solutions for the next binding series.

### 2.5. Cell cultures, incubation with inhibitors and assay of phosphatase activity

B50 (neuroblastoma), H9C2 (cardiomyoblast) and HepG2 (hepatocarcinoma) cell lines were obtained from the European Collection of Cell Cultures, and cultured according to the supplier's