A strategy for designing "multi-prong" enzyme inhibitors by incorporating selective ligands to the liposomal surface†

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We offer a novel strategy for designing "multi-prong" inhibitors of enzymes by incorporating selective ligands on the liposomal surface.

With explosion of biomedical research in recent genomics and the proteomics era, the number of potential drug targets has considerably increased.[1] This has prompted development of methodologies for rapid synthesis and screening of therapeutic agents against high priority targets for controlling human diseases. For pathogenic enzymes, both combinatorial and rational approaches have been utilized from time to time.[2] Irrespectively, once a lead enzyme inhibitor molecule is identified, there is always a need for fine tuning its structure so as to achieve an enhanced potency as well as selectivity for the target enzyme, and these features have been one of major challenges for medicinal chemists. This is primarily because of the lack of predictability whether a particular change in the ligand structure of the lead compound would be tolerated within the enzyme's active site, yielding a highly potent and selective inhibitor for the enzyme. To circumvent such a problem, we[3] and others[4] explored the possibility of designing enzyme inhibitors, which would not only bind to the active site pockets of enzymes but also to their surface-exposed residues. With the precedent that benzenesulfonamide serves as an active site directed (albeit weak) inhibitor for carbonic anhydrases[5] and matrix metalloproteinases[6] and that iminodiacetate (IDA)-Cu^{2+} exhibits the potential to selectively interact with the surface histidine residues of proteins at neutral pH,[7] we designed a series of "two-prong" inhibitors of these enzymes by attaching the above moieties *via* different chain length spacers. Such inhibitors exhibited much tighter binding affinities for selected carbonic anhydrase isozymes than that given by the parent (active site directed) inhibitor, benzenesulfonamide.[3] The binding modes of one such "two-prong" inhibitor to human carbonic anhydrase isozymes I and II have been revealed by the recent X-ray crystallographic studies, and the structural data clearly provide the molecular basis of the "two-prong" effect.[8]

Although our preliminary data appeared promising, the employment of IDA-Cu^{2+} containing multi-prong ligands for achieving selective inhibition of one enzyme as opposed to the other was found to be challenging. Besides intrinsic flexibility and steric constraints posed by the IDA-Cu^{2+} arms, fine-tuning of ligand structures to achieve desired selectivity appeared to be difficult. To circumvent such a limitation, we conceived an alternative approach of mimicking "multi-prong" inhibitor for enzymes by incorporating enzyme selective ligands onto the liposomal surface (Fig. 1).

Although there are few reports on the liposome/lipid mediated detection and desensitization of proteins/receptors,[9] they are not extendable toward rationally designing "multi-prong" inhibitors for any enzyme/protein of choice (see below). Our approach relies on the initial anchoring of the target enzyme on to the liposomal surface *via* the active site specific (albeit weak) ligand. Once the enzyme is bound to the liposomal surface, lipid mobility facilitates complementary interaction between secondary head groups and the cognate surface-exposed residues of the enzyme (Fig. 1), resulting in the liposome-based "multi-prong" inhibition of the enzyme. This approach, in principle, can be envisaged to circumvent the complex synthetic protocols, as well as fine tuning of lead structures of purely synthetic compounds to serve as "two" or "multi-prong" inhibitors for enzymes. As long as the active and peripheral site directed ligands can be incorporated as the lipid head groups, and the major lipid component is mobile (*i.e.*, predominates in the liquid phase) at room temperature, our liposome based strategy is likely to work with any enzyme system.

To establish the proof of our concept, we selected recombinant human carbonic anhydrase II (hCA II) as the target enzyme, and benzenesulfonamide and IDA-Cu^{2+} as the active site and surface (histidine) directed ligands for the enzyme, respectively. This

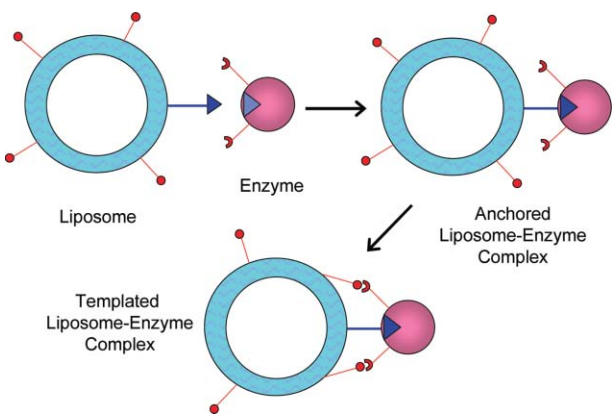

Fig. 1 A cartoon showing the multi-prong inhibition of an enzyme by incorporating selective ligands on the liposomal surface.

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system was chosen since we had previously demonstrated that the inhibitory potency of benzenesulfonamide was increased by 1–2 orders of magnitude when the former was conjugated with IDA-Cu^{2+} in two-prong inhibitors.[3] We prepared liposomes with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) as major (85–95%) component ($T_m = -2$ °C),[10] and linoleic acid conjugated benzenesulfonamide (BS-lipid) and stearic acid-conjugated IDA-Cu^{2+} lipid (Fig. 2) as minor (5–10%) components. In this preparation, the lipid components were deemed to be mobile at room temperature, and thus the interacting head groups could form complementary interactions with the cognate groups of the enzyme.

Fig. 3 shows the time courses of the hCA II catalyzed reactions utilizing *p*-nitrophenyl acetate as the esterolytic substrate in the absence and presence of differently formulated liposomes. Note that the reaction trace in the presence of the liposomes containing only IDA-Cu^{2+} as the inhibitor-head group is similar to that obtained in the absence of liposomes, suggesting that the presence of IDA-Cu^{2+} moiety alone has negligible effect on the rate of the enzyme catalysis. This is not surprising since we previously noted that although IDA-Cu^{2+} binds to the surface exposed histidine residues of the enzyme, it does not, by itself inhibit the catalytic activity,[3] presumably due to the fact that the Zn^{2+}-coordinated histidine residues (H94, H96, H119), present at the active site of the enzyme, do not interact with IDA-Cu^{2+} and consequently do not inhibit the enzyme. When liposomes containing only BS as inhibitor head group were included in the reaction medium, the catalytic rate of the enzyme was slightly impaired, as evident by a significant decrease in the slope of the kinetic trace (Fig. 3). The most pronounced diminution of the catalytic rate of the enzyme was noted when the liposomes containing both BS-lipid and IDA-Cu^{2+} lipid were added to the reaction medium. Clearly, the presence of benzenesulfonamide and IDA-Cu^{2+} (as lipid head groups) exhibited a synergistic effect in binding to the enzyme and thus impairing its catalytic activity. With precedent of our previous demonstration,[3,8] we conjecture that the origin of the above marked inhibitory feature lies in the concerted binding of benzenesulfonamide and IDA-Cu^{2+} groups at the active site and surface histidine residues of the hCA II, respectively.

To quantitate the magnitude of cooperation/coordination between benzenesulfonamide and IDA-Cu^{2+} moieties (when present on the same liposomal surface) on inhibition of hCA II, we performed the detailed steady-state kinetic experiments. Since both benzenesulfonamide and IDA-Cu^{2+} head groups are expected to be distributed between both outer and inner leaflets

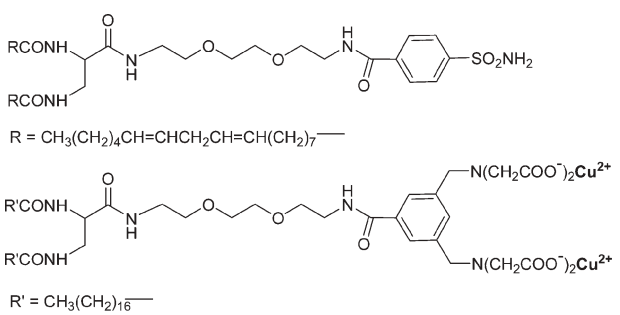

Fig. 2 Structures of the benzenesulfonamide and IDA-Cu^{2+} lipids used in the liposomal formulations.

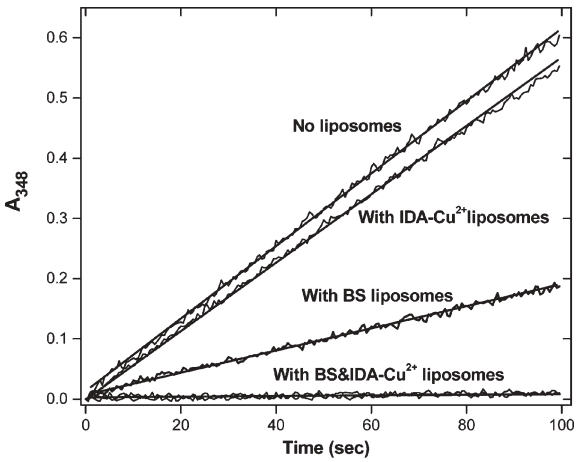

Fig. 3 Time courses of carbonic anhydrase catalyzed reaction in the absence and presence of differently formulated liposomes.

of liposomes, we estimated the effective concentrations of these species on the outer leaflet[11] in calculating the inhibition constants (K_i) of the enzyme–liposome complexes. Table 1 summarizes the K_i values of differently formulated liposomes for hCA II. The K_i value of aqueous benzenesulfonamide for hCA II is included in the Table for comparison. The data of Table 1 show that whereas the liposomes formulated with POPC alone have no inhibitory effect on hCA II catalysis, those that include BS and/or IDA-Cu^{2+} lipids exhibit different degrees of inhibitory potency. The fact that the K_i value of aqueous BS (1.5 μM) is similar to that of liposomes incorporating only BS as inhibitor head group (1.7 μM) suggests that constraining BS alone to the liposomal surface has no influence on its potency. However, the K_i value is drastically decreased from 1.7 μM to 70 nM, when the liposomes contain IDA-Cu^{2+} lipid as well. Clearly, the presence of both BS and IDA-Cu^{2+} lipids in the liposomes results in a marked decrease in the K_i value, and such an effect is due to "multi-prong" attachment of the above groups to the enzyme. However, it should be pointed out that even the presence of IDA-Cu^{2+} group on the liposomal surface exhibits some inhibitory effect (albeit about two orders of magnitude lower than that observed with the IDA-Cu^{2+} and BS containing lipids) presumably due to "aggregation" of ligands as elaborated by Schoichet and his collaborators.[12]

To ascertain whether the liposome mediated inhibition of hCA II was influenced by the presence of other proteins of the physiological system, we performed the above steady-state kinetic experiments in the presence of 10 and 30% bovine serum (see ESI†). The experimental data revealed that the presence of bovine serum had practically no influence on the liposome-dependent inhibition of the enzyme. Evidently, the presence of other proteins in bovine serum did not compete against hCA II for the binding

Table 1 Summary of inhibition constants (K_i)

Liposome composition	K_i/μM
Free BS	1.5 ± 0.1
POPC	No inhibition
POPC + BS-lipid (95 : 5)	1.7 ± 0.2
POPC + IDA-Cu^{2+} lipid (90 : 10)	150 ± 30
POPC + BS-lipid + IDA-Cu^{2+} lipid (85 : 5 : 10)	0.070 ± 0.032

with the BS and IDA-Cu^{2+} harbouring liposomes, presumably due to synergistic as well as selective effects in stabilizing the enzyme–inhibitor complex.

Admittedly, the initial syntheses and purification of selected lipid conjugates, and incorporating them into liposomes to generate multi-prong inhibitors can be challenging. However, once the overall protocol is standardized, the resultant liposomes can be utilized for desensitizing a wide range of enzymes. This will serve as an alternative of purely synthetic approach in designing multi-prong inhibitors for inhibiting the target enzymes with high potency and selectivity.

This is the first demonstration (to the best of our knowledge) of "rational" design of the liposome based "multi-prong" inhibitor for the inhibition of a model enzyme, human carbonic anhydrase II (hCA II), and the overall approach can be easily extended for inhibition/desensitization of other pathogenic enzymes, receptors, virus particles, among others. It should be emphasized that our approach is fundamentally different from those available in literature (usually involving one type of ligand head group) for desensitization of proteins/enzymes *via* statistically matching the patterns between proteins and liposomal surface.[9] The uniqueness of our approach lies in initially "luring" an enzyme to the liposomal surface, followed by strengthening the enzyme–liposome interaction (facilitated *via* the lipid mobility) between the secondary ligands (present as the lipid head groups) and the cognate surface residues of the enzyme. Although we have used IDA-Cu^{2+} as the secondary lipid head group to interact with the surface exposed histidine residues of hCA II,[3] the latter can be easily replaced by other positively or negatively charged head groups with comparable results. Our overall approach can be further fine-tuned toward designing liposome based "artificial antibodies" by locking the conformations of liposomes *via* photo-polymerization, which will find applications in the diagnostic area.

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