Organic & Biomolecular Chemistry

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Cite this: *Org. Biomol. Chem.*, 2012, **10**, 40

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# Anion mediated activation of guanidine rich small molecules†

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***Received 11th August 2011, Accepted 26th September 2011***
**DOI: 10.1039/c1ob06373a**



**Cell penetrating peptides (CPPs) and their synthetic analogs are of widespread interest. Here we report that guanidine rich small molecules can be potential membrane transporters in the presence of hydrophobic counteranion activators. To our knowledge, this is the first example of small molecules that mimic the anion-activated transport function of CPP.**

Cell penetrating peptides (CPPs) and their synthetic analogs are of widespread interest as they show the remarkable ability to transverse membranes despite their high charge density.[1] They also show promise as unique delivery vehicles. Most CPPs are rich in arginines; arginines are well known to exist with tightly bound but exchangeable counteranions. According to Matile and co-workers this counteranion scavenging may be how guanidine rich molecules minimize intramolecular charge repulsion.[2] The weak acidity of the guanidinium group hinders partial deprotonation unlike free amine groups under physiological condition.[1g] Their transduction activity has also been linked to this counterion scavenging.[1g] It has been proposed that guanidine rich CPPs can alter their solubility upon counteranion exchange with more or less polar anions allowing them to adapt to their environment and cross the lipid bilayer.[3] It appears that neutralization of the charge is not essential assuming the guanidine rich molecules have other hydrophobes in their structure. This ability to incorporate hydrophobic activators into the molecular structure was termed 'self-activation'.[1o]

Early on Rothbard and co-workers showed that polyamides, mostly peptides, entered cells in a length dependent fashion. Trimers, tetramers, and pentamers hardly entered Jurkat cells while hexamers showed significantly more internalization.[1i,1k] Intracellular staining increased from hexamers to nonamers. Similar results have been observed for other CPPs including HIV-TAT derivatives; the nona-peptide $TAT_{49-57}$ efficiently translocated into Jurkat cells while shorter (truncated) analogs were much less active.[1i,4] Small biphenyl-based molecules with two and four guanidines were shown to enter human U2OS osteosarcoma cells, but the derivative with four guanidines worked better than the derivative with two.[5] More recently, a number of synthetic polymers were shown to effectively mimic CPPs.[1l–o,6] In general, these papers reported molecules with more than eight repeat units, except for one case in which a pentamer was studied but this molecule contained ten guanidine units.[1l–o,4b,6] Given the widespread interest in CPP derivatives, the ability to design small guanidine rich molecules capable of transversing the plasma membrane would be exciting. Here, two guanidine rich molecules, **CPPM1** and **CPPM2**, were designed to evaluate their transport activity (see Fig. 1).

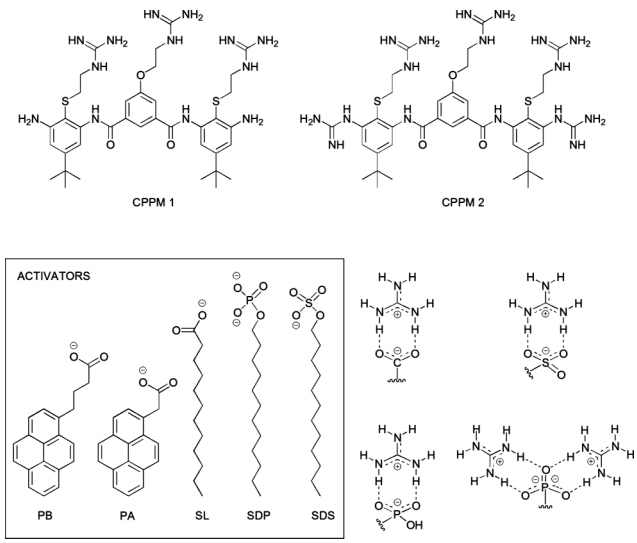


**Fig. 1** Cell penetrating peptide mimics (CPPMs) and the hydrophobic counteranion activators used in this study. The possible hydrogen bonding structures are shown between CPPM's guanidines and the activator's anions.

Model membrane studies were specifically chosen for two reasons. First, to study cellular internalization it is common to covalently attach a dye to the transporter. For these small molecules, a dye molecule would be of similar size and expected to have a significant influence on its uptake properties. Second, uptake into cells can involve a number of mechanistic pathways and we remain fundamentally interested in 'passive diffusion' or non-energy driven mechanisms. Of course cellular uptake studies are critical if delivery of therapeutics is to be achieved but that was not the goal of the current study. This study aimed to understand whether these two small oligomers effectively transverse plasma membranes by themselves or required assistance from hydrophobic counteranions.

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† Electronic supplementary information (ESI) available: Liposome preparation, fluorescence assay, transporter activity with counteranions, statistical analyses. See DOI: 10.1039/c1ob06373a

**CPPM1** consists of three guanidine groups and two amine groups in the side chains while **CPPM2** contains five guanidine groups. This design provided the direct study of guanidine density on transport. At the same time, the role of the primary amine groups on anion mediated activation in these derivatives could be studied. **CPPM1** and **CPPM2** were synthesized in a few routine steps from commercially available starting materials as reported previously.[7] Five amphiphilic anions were selected to evaluate their ability to activate these CPPMs (see Fig. 1). Pyrenebutyrate (PB) and pyreneacetate (PA) were chosen as they are the most commonly used aromatic activators for polyarginines (**pR**).[8] In fact, PB was previously found to be the best activator for CPP mediated delivery.[8b,9] To directly investigate the effect of various anions in activation, three aliphatic anions containing an equal number of carbon atoms, sodium laurate (SL), sodium dodecyl phosphate (SDP), and sodium dodecyl sulfate (SDS) were picked from the known collection of aliphatic counteranion activators.[2,8a]

In order to assess the ability of these CPPMs to transverse mammalian-like membranes, neutral phosphatidylcholine large unilamellar vesicles (EYPC-LUVs) were prepared and used in the classical 5(6)-carboxyfluorescein (CF) assay, well accepted in the CPP field.[1e,1m,8a,10] In this assay the release of self quenched CF from EYPC-LUVs⊃CF is monitored continuously as an increase of fluorescence intensity against time. As shown in Fig. 2a and 2d (red curves), neither CPPM showed significant activity in the absence of hydrophobic counteranions. However, addition of these counteranions had the expected activating effect as measured by increased fluorescence intensity.

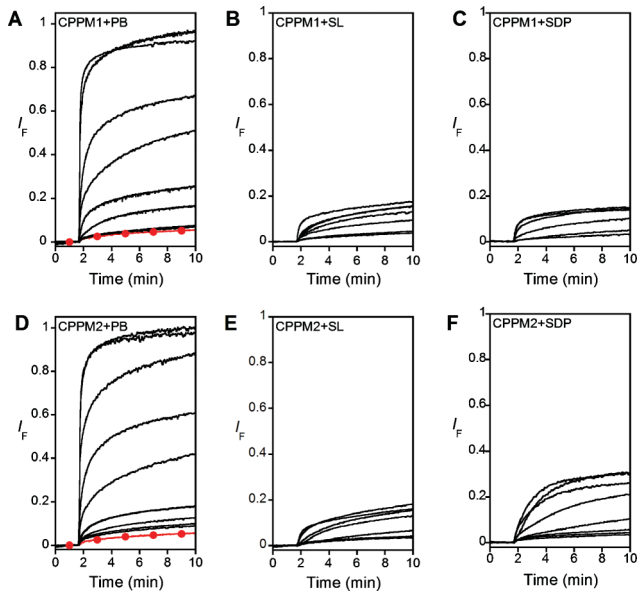


**Fig. 2** Changes in CF emission ($I_F$) ($\lambda_{ex}$ 492 nm, $\lambda_{em}$ 517 nm) as a function of time with increasing activator concentration [0 (red curve), 0.1, 1, 5, 10, 20, 25, 50, 100 μM PB in A; 1, 5, 10, 30, 60, 90, 180 μM SL in B; 1, 10, 30, 60, 90, 120 μM SDP in C; 0 (red curve), 0.1, 0.5, 5, 10, 20, 25, 50, 75, 100 μM PB in D; 5, 10, 30, 60, 90, 120, 180, 300 μM SL in E; 0.1, 1, 5, 10, 20, 30, 60, 120 μM SDP in F] during addition of **CPPM1** (2.5 μM, final concentration) or **CPPM2** (2.5 μM, final concentration) at $t$ = 100 s to EYPC-LUVs⊃CF (50 μM EYPC), calibrated by final analysis ($I_F$ = 1.0, with 40 μL 1.2% aqueous triton X-100).

The activity of the CPPMs increased with increasing activator concentration at a constant CPPM concentration and constant lipid concentration, yielding plots of fractional fluorescence intensity ($I_F$) *versus* time for different activator concentrations (Fig. 2). Fitting the Hill equation, $Y \propto (c/EC_{50})^n$, for each individual activator revealed a nonlinear dependence of the fractional activity, $Y$, on the activator concentration, $c$. This analysis gave $Y_{max}$ (maximal CF release relative to complete release by Triton X-100), $EC_{50}$ (effective activator concentration needed to reach $Y_{max}/2$), and $n$ (the Hill coefficient).

Fig. 3 and Table 1 collects the $EC_{50}$ and $Y_{max}$ values for these two CPPMs with the various activators at a constant CPPM/lipid (P/L) ratio 0.05 (guanidine to lipid ratio, 0.15 and 0.25 for **CPPM1** and **CPPM2** respectively). Consistent with previous reports of **pR** activation,[2,8a] the two aromatic activators (PB and PA) gave the highest $Y_{max}$ values for these CPPMs. PB had an $EC_{50}$ approximately 3.5 times lower than PA for both **CPPM1** and **CPPM2** indicating this hydrophobic, aromatic anion activator is more effective. The three aliphatic activators had considerably lower $Y_{max}$ values. From Table 1, it is clear that these activators fall into two categories based on their $Y_{max}$ values in which the aromatic anions are more effective at inducing CF transport out of the vesicles (also see Fig. 3 and Supplementary Information, SI, Figure S2†). This highlights a problem with how $EC_{50}$ values should be compared. When all activators give similar $Y_{max}$ valves, the comparison of $EC_{50}$ is straightforward and meaningful. Therefore, from Table 1, it is easy to compare the $EC_{50}$'s of PB to PA and SL to SDS to SDP.

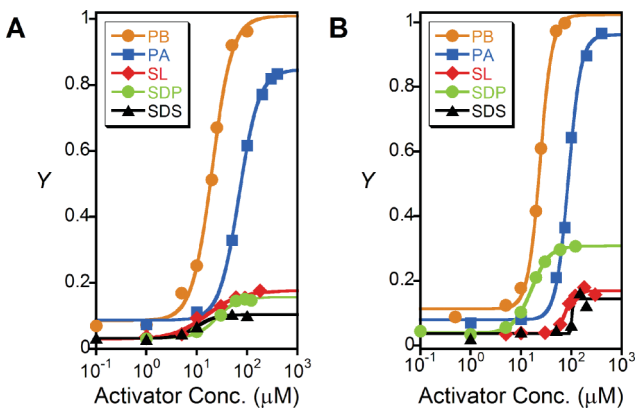


**Fig. 3** Dose response curves for PB, PA, SL, SDP, and SDS with (A) **CPPM1** (2.5 μM) and (B) **CPPM2** (2.5 μM) against EYPC-LUVs⊃CF vesicles (50 μM EYPC), with curve fit to Hill equation.

However, when the $Y_{max}$ values differ significantly meaningful comparisons of $EC_{50}$ are less obvious. A perfect activator would have a maximum $Y_{max}$ and low $EC_{50}$.[2] Previous literature with a classical CPP, **pR**, at the P/L 0.02 (guanidine to lipid ratio 1.4) showed a general trend of decreasing $EC_{50}$ accompanied by decreasing $Y_{max}$ for a group of activators; not very promising for potent activator selection.[2,8a] Fortunately, Matile and co-workers proposed a solution to this activator comparison dilemma by creating a term called activator efficiency, $E$, based on the exponential relationship between $Y_{max}$ and $EC_{50}$, with a scale between 0 and 10.[2] This term is also helpful since it allows experiments to be compared when the concentrations of the activators and/or transporters are different between experiments.

**Table 1** $EC_{50}$, $Y_{max}$, and $E$ values for anion activators in EYPC-LUVs⊃CF at constant lipid concentration (50 μM) and CPPM concentration (2.5 μM)

| | CPPM 1 | | | CPPM 2 | | | pR[a] | | |
|---|---|---|---|---|---|---|---|---|---|
| Activator | $EC_{50}$ (μM) | $Y_{max}$ | $E$ | $EC_{50}$ (μM) | $Y_{max}$ | $E$ | $EC_{50}$ (μM) | $Y_{max}$ | $E$ |
| PB | 20 ± 1.3 | 1.0 ± 0.04 | 8.2 | 24 ± 0.8 | 1.0 ± 0.02 | 7.9 | 44 ± 2.0 | 0.78 ± 0.02 | 5.1 |
| PA | 70 ± 2.4 | 0.85 ± 0.01 | 4.8 | 88 ± 3.6 | 0.96 ± 0.03 | 4.9 | 86 ± 3.0 | 0.80 ± 0.03 | 4.1 |
| SL | 15 ± 2.0 | 0.17 ± 0.02 | 1.5 | 76 ± 4.3 | 0.17 ± 0.006 | 0.9 | 34 ± 1.0 | 0.10 ± 0.01 | 0.7 |
| SDP | 25 ± 3.1 | 0.15 ± 0.008 | 1.2 | 16 ± 0.4 | 0.3 ± 0.004 | 2.6 | 19 ± 1.0 | 0.61 ± 0.03 | 5.1 |
| SDS | 10 ± 1.0 | 0.10 ± 0.003 | 1.0 | 103 ± 0.02 | 0.14 ± 0.02 | 0.7 | 16 ± 1.0 | 0.27 ± 0.01 | 2.4 |

$Y_{max}$ (maximal CF release relative to complete release by Triton X-100); $EC_{50}$ (effective polymer concentration needed to reach $Y_{max}/2$); $E$, activator efficiency. Each data point was collected in three independent experiments.[a] pR activation data incorporated from refs. 2, 8a.



Table 1 also shows $E$ values for each activator with **CPPM1**, **CPPM2**, and literature values of **pR**. Not surprisingly, PB is the best activator for all three of these guanidinium-rich transporters. It even activates the two small oligomers better than **pR**. The phosphate containing activator, SDP, also performed well for **pR** and **CPPM2**. Both of these molecules contain only guanidinium cations which are known to form strong and unique interactions with phosphate anions (see Fig. 1). In fact, comparing activation of **CPPM1** and **CPPM2** with SDP shows that the two extra guanidines present in **CPPM2** yield a lower $EC_{50}$ and higher $Y_{max}$ (see SI, Figure S2† for statistical analyses), or overall better activation ($E$ = 2.6 *vs.* 1.2). This is consistent with the ability of phosphate ions to form specific interactions with guanidinium over amines.[1d,3] Further, aromatic counteranion activation of both CPPMs in EYPC vesicles is more selective than **pR** as shown by comparing the $E$ values of PB *vs.* PA.

Comparing the two non-phosphate aliphatic anions shows that these two activators, SL and SDS, are more effective for **CPPM1** than **CPPM2** (Table 1). For example, SL gives identical $Y_{max}$ values for both CPPMs but the $EC_{50}$ of **CPPM1-SL** is five times lower than **CPPM2-SL** (also see SI, Figure S2† for statistical analyses). This suggests an important role for the amines present in **CPPM1**. Lehn *et al.* reported that the less-delocalized, harder amine cation binds anions more strongly even though guanidinium had a prefer geometry to form bi-dentate interactions.[11] It thus seems likely that the similarly less delocalized carboxylate can interact with the amines of **CPPM1**.

In summary, this paper demonstrates the ability of common hydrophobic anions to serve as activators for two guanidinium-rich small molecules. Although the detailed mechanism is unknown and beyond the scope of this communication,[12] the results confirm the ability of these small molecules to serve as transporters through model membranes. It re-confirms the special role aromatic anions, like pyrenebutyrate, have in facilitating transport. Comparing activator efficiencies to the well-studied polyarginine shows that PB activates these small molecules better. Comparing **CPPM1** to **CPPM2** showed that the amine groups allowed better activation by the aliphatic carboxylate anion. This strategy can facilitate guanidine rich small molecule mediated cargo delivery and suggests small molecules may present a rich new opportunity to design novel CPPMs.

## Acknowledgements

This work was supported by a NSF grant (CHE-0910963) to GNT.

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