Phosphazenylyphosphonium Frameworks: A Novel Main Group Mitochondrial Delivery Vector with Enhanced Properties


Abstract: Mitochondrial targeting represents an attractive strategy for treating metabolic, degenerative and hyperproliferative diseases, since this organelle plays key roles in essential cellular functions. Triphenylphosphonium (TPP+) moieties – the current “gold standard” – have been widely used as mitochondrial targeting vectors for a wide range of molecular cargo. Recently, further optimisation of the TPP+ platform drew considerable interest as a way to enhance mitochondrial therapies. However, although the modification of this system appears promising, the core structure of the TPP+ moiety remains largely unchanged. Thus, in this study, we explored the use of aminophosphonium (PN+) and phosphazenylyphosphonium (PPN+) main group frameworks as novel mitochondrial delivery vectors. The PPN+ moiety was found to be a highly promising platform for this purpose, owing to its unique electronic properties and high lipophilicity. This has been demonstrated by the high mitochondrial accumulation of a PPN+-conjugated fluorophore relative to its TPP+-conjugated counterpart, and has been further supported by density functional theory and molecular dynamics calculations, highlighting the PPN+ moiety’s unusual electronic properties. These results demonstrate the potential of novel phosphorus-nitrogen based frameworks as highly effective mitochondrial delivery vectors over traditional TPP+ vectors.

Introduction

The ability to deliver compounds selectively to the mitochondria is rapidly growing in importance due to the increasing relevance of the organelle in disease treatment.[1] Mitochondria-selective accumulation of compounds can be accomplished by some cationic moieties commonly known as delocalised lipophilic cations (DLCs),[2] and this ability is frequently attributed to their delocalised cationic charge, resulting in increased lipid membrane permeability and accumulation within the negatively-charged mitochondrial matrix in accordance with the Nernst equation.[2c, 3] While there are a broad range of mitochondrial targeting species, from rhodamines, cyanines, mitochondrial-targeting peptides to thiophene-based vectors,[2a, 4] triphenylphosphonium-based vectors are arguably the most widely used and have been applied for the delivery of numerous molecular cargoes as highlighted in the literature.[5] Due to the success in applying triphenylphosphonium (TPP+) systems for mitochondrial delivery, there is an increasing interest in modifying the TPP+ systems for enhanced delivery.[6]

Although they are often referred to as DLCs,[2b, 7] in the context of TPP+ systems, the necessity of charge delocalisation for mitochondrial accumulation is ambiguous. In contrast with DLCs such as rhodamines and cyanines, TPP+ moieties do not typically contain polyaromatic or extended conjugated moieties. While studies show that modification of the TPP+ moiety can result in improvements of biological activity, few focus on the electronic
Herein we present the synthesis and characterisation of PN\(^+\) and PPN\(^+\) salts, and their evaluation as improved novel mitochondrial delivery vectors. The enhanced ability of the PPN\(^+\) moiety to deliver a fluorescent cargo selectively to the mitochondria was also demonstrated and was shown to be superior to the "gold standard" TPP\(^+\) moiety. The main reason for this behaviour is attributed to the more extensive charge delocalisation of the PPN\(^+\) salts, as demonstrated here through the calculation of electrostatic potential surface (ESP) maps for the studied compounds. This work seeks to provide insights into the effects of charge delocalisation on the physical and biological properties of these compounds, as well as demonstrate the efficacy of the PPN\(^+\) framework as a powerful tool for mitochondrial delivery.

**Results and Discussion**

**Synthesis and characterisation**

Initial screening of the uptake was conducted using the new phosphonium vectors with a short alkyl chain as a model compound (Figure 1, compounds 1–3). The synthetic pathway is summarised in Figure 2B (Routes a–c). The synthesis of the TPP\(^+\) control (1) was achieved by reacting triphenylphosphine with ethyl bromide (Route a).\(^{[6]}\) The aminotriphenylphosphonium vector (2) was synthesised through the bromination of triphenylphosphine, followed by treatment with methylamine in the presence of triethylamine (Route b).\(^{[14]}\) The PPN\(^+\) vector (3) was synthesised via a two-step process (Route c): 1) bromination of triphenylphosphine followed by bubbling ammonia through the mixture to obtain aminotriphenylphosphonium bromide, 2) double deprotonation of the amino protons using n-butyl lithium, treatment with diphenylchlorophosphine and ethyl bromide.\(^{[15]}\) All three compounds were characterised via NMR spectroscopy (\(^{1}H,^{13}C,^{31}P\)), HRMS and single-crystal XRD studies. The NMR and HRMS spectra were consistent with the proposed structures (See ESI).

![Figure 2. A) Canonical structures of compound of aminophosphonium (PN\(^+\)) 2 and phosphazeny phosphonium (PPN\(^+\)) 3 cations, left and right respectively. B) Synthesis of compounds 1 – 5.](image-url)
The X-ray solid-state structures obtained present a shortened P-N bond length in both 2 and 3 – 1.625 and 1.582 Å, respectively – which are shorter than the typical phosphazane P-N. Notably, the bond angle of the P-N-C fragment in 2 was 119.22° and closely matched the expected sp² geometry. Compound 3 showed a bent P-N=P geometry, with an increased bond angle of 139.70°, consistent with known compounds with a P-N=P backbone. Interestingly, despite the co-crystallization of compound 3 with a water molecule, there were no short contacts observed between the water molecule and the P-N=P backbone, highlighting the weakly-coordinating nature of the cation.

The DFT optimised structures of compounds 2 and 3 for the subsequent computational studies, showed that the P-N bond length was of 1.65 Å and 1.60 Å, respectively. In addition, the P-N-C and P-N-P angles were 126.21° and 132.88°. Furthermore, an NBO-charge analysis on compound 3, supported a dication bridged by a negatively charged nitrogen – with P and N atomic charges of 1.862 a.u. and -1.462 a.u., respectively. These results were thus in line with the experimental characterisation of compounds 2 and 3.

**Cytotoxicity and Lipophilicity**

Due to the high accumulation of lipophilic cations within the mitochondria, DLCs induce mitochondrial membrane depolarisation at high concentrations – which causes cell death. Thus, cytotoxicity values can be used as a surrogate to measure mitochondrial accumulation in vivo.

In this study, cytotoxicity assays using HeLa cells were carried out to obtain the 72h IC₅₀ values. The results obtained are summarised in Table 1. Relative to the TPP⁺ control, compound 2 showed an increase in the IC₅₀ values from 16.15 µM to 46.36 µM, indicating poorer performance. On the other hand, compound 3 has a drastically lowered IC₅₀ at 0.77 µM. To rationalise these findings, the water-octanol partition coefficient (logP), was experimentally obtained via an HPLC method reported in the literature and summarised in Table 1. LogP, frequently used as a measure of lipophilicity, is a critical parameter in biological systems. Quantitative Structure-Activity Relationship (QSAR) models for mitochondrial uptake and has been found to be particularly relevant in TPP⁺ systems.

According to QSAR models developed by Horobin et. al, logP is required to fall between 0 to 5 for optimal mitochondrial uptake. It has been previously established that an increase in logP is generally well correlated with an increase in mitochondrial accumulation. Relative to compound 1 (logP = -1.36), – the current “gold-standard” – compound 2 had a marginally higher logP of -1.28, which goes against the expected trends. We attributed this discrepancy to the increased ion-pairing tendency of this compound (vide infra). Compound 3, on the other hand, had a logP of 0.51, which is within QSAR the range for optimal mitochondrial accumulation. This is consistent with the lowered IC₅₀ observed, indicating that the PPN⁺ compounds are potentially highly efficient mitochondrial vectors.

The apparent discrepancy between IC₅₀ and logP for compound 2 was attributed to the increased formation of an ion-pair for compound 2 due to the introduction of the solvent-exposed N-H hydrogen bond donor (HBD). LogP measurements conducted in phosphate-buffered saline for 1 and 2 revealed an apparent increase in lipophilicity for both compounds (from -1.36 to -0.429 and -1.28 to 0.527, respectively, see Table S1). The higher logP in a solution with increased salt concentration is expected due to the increased counterion concentration. However, the relative increase in lipophilicity was significantly larger in 2, indicating a stronger influence from the high salt concentrations. The increased tendency to form ion-pairs due to the presence of the solvent-exposed HBD moiety renders compound 2 as an inefficient mitochondrial delivery vector since the ion-pair is electrically neutral.

**Table 1. Experimental and computational data for compounds 1 – 5.**

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀ (± 95% CI) / µM</th>
<th>logP (± SD)</th>
<th>logP_monom (± SD)</th>
<th>V_mol / kcal∙mol⁻¹</th>
<th>SASA / nm²</th>
<th>Volume / nm³</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16.15 ± 1.29</td>
<td>-1.36 ± 0.09</td>
<td>-0.73 ± 0.01</td>
<td>96.15</td>
<td>5.381 ± 0.189</td>
<td>0.891 ± 0.026</td>
</tr>
<tr>
<td>2</td>
<td>46.36 ± 5.43</td>
<td>-1.28 ± 0.01</td>
<td>-0.70 ± 0.06</td>
<td>117.36</td>
<td>5.340 ± 0.196</td>
<td>0.877 ± 0.027</td>
</tr>
<tr>
<td>3</td>
<td>0.77 ± 0.27</td>
<td>0.507 ± 0.03</td>
<td>-0.53 ± 0.20</td>
<td>80.20</td>
<td>7.312 ± 0.219</td>
<td>1.323 ± 0.033</td>
</tr>
<tr>
<td>4</td>
<td>6.90 ± 1.94</td>
<td>-1.29 ± 0.194</td>
<td>-0.74 ± 0.02</td>
<td>88.86</td>
<td>5.451 ± 0.185</td>
<td>0.912 ± 0.027</td>
</tr>
<tr>
<td>5</td>
<td>8.98 ± 1.52</td>
<td>-1.28 ± 0.01</td>
<td>-0.69 ± 0.07</td>
<td>94.79</td>
<td>5.475 ± 0.190</td>
<td>0.920 ± 0.027</td>
</tr>
</tbody>
</table>
To eliminate the increased ion pairing potential from the N-H bond, two additional compounds were synthesised – an aminophosphoniu salt with a tertiary amino group, together with the alkyl substituted counterpart as a control (i.e., NMe₂ and -CHMe₂, compounds 4 and 5, respectively – see Figure 1).[19] The cytotoxicity assays revealed that the IC₅₀ values for these two compounds are 6.90 µM and 8.98 µM, respectively, in line with our hypothesis – see Table 1. This is further supported by the single-crystal structures, with the presence of short contacts between the P/P-N moiety and the bromide counterion only observed in compound 2 (NH···Br = 2.445 Å) and absent in all other compounds studied (compounds 1, and 3 – 5) – see Figure 3 and ESI. Although a slight improvement of IC₅₀ is observed for compounds 4 and 5 with respect to 2, the differences are not sufficiently significant for subsequent biological studies. Therefore, further studies on PN system were not pursued.

The evidence obtained during our quest for non-conventional mitochondrial delivery vectors highlights the limitations of lipophilicity as a parameter for mitochondrial accumulation when applied to DLCs. This limitation is especially pronounced when a large difference in hydrogen bonding ability is present between the series compounds studies, as logP measures the lipophilicity of the ion pair, while mitochondrial targeting ability depends largely on the cationic moiety.

**Structural and computational studies**

To further understand the influence of molecular modifications on electronic properties, charge distribution, and lipophilicity, we performed a molecular dynamics study and quantum mechanical calculations for compounds 1 – 5. In line with previous works, the molecular volume (Vol), solvent-accessible surface area (SASA), electrostatic surface potential (Vs), and membrane translocation free energy profiles were calculated.

One of the most promising parameters for the studies series was the maximum of the molecular electrostatic potential surface of the cations, Vₛ,max – represented in Figure 4. More positive Vₛ,max values have been related to stronger halogen/hydrogen bonding-anion intermolecular interactions.[20] The charge distribution at the molecular surface (delocalisation and shielding) has also been related to the ability of DLCs to cross hydrophobic membranes.[6a] However, to the best of our knowledge, this parameter was never used to rationalise the mitochondrial uptake of these species.

When correlating Vₛ,max values with the IC₅₀ results for compounds 1 – 5, a very significant linear correlation was observed (r²=0.95, see ESI, Figure S3). This could indicate that Vₛ,max might be used as a parameter of mitochondrial vector ability. We have then increased the compound dataset to include previously tested cations (including dications) from our previous reports (compounds 8 – 23 in Figure S4 and Table S4).[6a, 6d, 6f] This enlarged dataset made up a total of 21 cations (16 additional compounds) with a series of experimental correlations shown in Figure S5.

The IC₅₀ and Vₛ,max results still display significative correlation, even though r² was lower (r²=0.46, and r²=0.55 excluding the dications in Figure S5a). Still, the overall trend for the cations with similar molecular features seemed to indicate that those that presented lower IC₅₀ had lower Vₛ,max. The lower, but significant r² values, might result from several factors: 1) some of the compounds might be approaching the upper limit for the lipophilicity-linked toxicity, with negligible changes in IC₅₀ over an order of magnitude of [TPP⁺];[6d] 2) the data lacks cations with IC₅₀ in the 50-150 µM range, and 3) by using a more diverse set of compounds, the correlation may become non-linear.

Nevertheless, it is evident that the Vₛ,max property is a valuable descriptor to differentiate compounds when charge delocalisation is significant (i.e. PN vs. PC systems). When delocalisation is not

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*Figure 4. Calculated electrostatic potential surfaces for cations 1 – 5, calculated at the wB97XD/6-311+G(d,p) level of theory. Maximum ESP values, Vₛ,max, are shown as a green sphere and their numerical value reported next to it.*
pronounced, or when approaching the lipophilicity-linked toxicity upper limit, accumulation properties may be better described by parameters based on molecular ion charge to volume ratios (i.e., charge density ratios, Z/Vol or Vol/Z).

Thus, we have calculated a non-linear relationship between Vol/Z and IC₅₀, which worked similarly to the logP vs IC₅₀ correlation (see Figure S5b). This similarity is consistent with the good linear correlation observed between logP and Vol/Z (r²=0.86, Figure S5c).

When Z/Vol is used instead, a good logarithmic correlation is observed with IC₅₀. Furthermore, if our compound series (excluding the dications) is divided into two regions (IC₅₀ < 3 μM and IC₅₀ > 3 μM), it can be observed that for the first region the linear correlation is better for the Z/Vol parameter than for Vₜₘₐₓ (r²=0.71 vs. r²=0.47, respectively, see Figure S5e). However, when moving to the region of IC₅₀ > 3 μM, the linear correlation is better with Vₜₘₐₓ (r²=0.92) than with Z/Vol (r²=0.31) – as shown in Figure S5f.

In addition, we studied the partition of cations 1 – 5 at a biological membrane model (100% POPC) using molecular dynamics simulations, to see if in a more realistic water:membrane system than water:octanol, small differences in the lipophilicity and translocation of the cations could be captured, which could better correlate with the toxicity IC₅₀ results. In Figure S1, we present the translocation free energy profiles of all five cations (1 – 5) across a water:POPC bilayer system. These profiles were then used to compute the membrane partition coefficients (logPₜₘₐₓ) by calculating the standard binding free energy of the cations to the membrane (see ESI for the full details).

The logPₜₘₐₓ values gave similar trends to the experimental logP results (see Table 1). More specifically, we obtained a very similar membrane partition for the TPP⁺ (1) and PN⁺ (2) cations (-0.73 ± 0.01 and -0.70 ± 0.06, respectively), and a higher membrane partition for the PPN⁺ (3) cation (-0.53 ± 0.20). As for the other two cations, 4 and 5, we have obtained very similar logPₜₘₐₓ results to 1 and 2 (-0.74 ± 0.02 and -0.69 ± 0.07, respectively). We also analysed other parameters taken from the free energy profiles, such as the free energy barrier at the centre of the bilayer (ΔGₐ).

However, none of these correlated linearly with the observed IC₅₀ results (see ESI, Table S2 and Figure S2). Despite the difficulty of computational methods in capturing some subtle effects, our calculations are remarkably in line with the experimental trends. They corroborate the limitations of lipophilicity as a parameter for predicting mitochondrial toxicity when applied to our sets of TPP⁺ molecules.

We then evaluated electronic and molecular properties of the cations that have been related to aqueous solubility, more specifically the SASA, Vol, HOMO-LUMO orbitals, isotropic polarizability, and dipole moment. The electronic properties were calculated at the DFT level using the software Gaussian 09 (see ESI for the full details). These properties were correlated with the lipophilicity and toxicity of the tested molecules, but again, they could not explain the toxicity IC₅₀ results of the five cations. However, they correlated well with the experimental logP values available (compounds 1 – 5). These results are presented in Table S3 (see ESI).

Finally, using a polarizable continuum solvent framework, we have assessed the propensity for ion-pair formation of two of the cations in the dataset (cations 1 and 2). In this regard, we have calculated the solvation free energy of the cations and the respective ion-pairs (with Cl⁻) in water and hexane (a model lipophilic liquid that represents here the inner part of the bilayer) – see Table S5 in the ESI).

The calculations show that for the ion-pairs comprised compounds cations 1 and 2, the energy of transfer from water to n-hexane is 64 kJ·mol⁻¹ and 38 kJ·mol⁻¹, respectively. In contrast, the transfer free energy between water and n-hexane for both cations was approximately the same (49 kJ·mol⁻¹). Thus, ion pairing leads to better charge neutralization, and less zwitterionic character in compound 2. An additional analysis of the partial charges on the ion-pair was performed to examine the charge delocalisation over the cation-anion complex for compounds 1 and 2. It was also observed that the partial charge on the chloride anion was less negative for the cation 2Cl⁻ complex when compared with the 1Cl⁻ ion pair (see Figure S6). This supports a higher charge delocalisation of the DLC for the 2Cl⁻ ion-pair, which could decrease the energy of the ion-pair in the hydrophobic phase. A similar effect, has previously been proposed anion-triazole ion pair complexes. In addition, these results are in accordance with the previous previously discussed logP and IC₅₀ experimental results observed for cation 2 (vide supra).

Confocal colocalization studies

To investigate the mitochondrial targeting ability of the PPN⁺ moiety, it was conjugated to a fluorescein derivative previously reported in the literature. However, to control regioselectivity and eliminate the protonophoric site, a methyl ester of fluorescein was employed to avoid mixtures of products. An analogous TPP⁺ variant was also synthesised as a control.

The structures of the synthesised conjugates are shown in Figure 1 (i.e., compounds 6 and 7). The logP of the dye conjugates were also measured experimentally (Table 2). As expected, the conjugation of the highly lipophilic PPN⁺ moiety resulted in a dye conjugate with a higher logP of 2.55, as compared to 0.417 in the TPP⁺ conjugate.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Fluo/MT (± SD)</th>
<th>logP (± SD)</th>
<th>Pearson’s coefficient</th>
<th>M1 (± SD)</th>
<th>M2 (± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>0.065 ± 0.05</td>
<td>0.417 ± 0.04</td>
<td>0.568 ± 0.108</td>
<td>0.954 ± 0.023</td>
<td>0.874 ± 0.013</td>
</tr>
<tr>
<td>7</td>
<td>0.666 ± 0.04</td>
<td>2.55 ± 0.10</td>
<td>0.887 ± 0.007</td>
<td>0.961 ± 0.011</td>
<td>0.971 ± 0.011</td>
</tr>
</tbody>
</table>

The results of the experimental data for compounds 6 and 7. Fluos/MT, Pearson’s and Mander’s coefficients (M1 and M2) refers to the intensity ratios, correlation, and co-occurrence between the fluorescein and MitoTracker channels respectively.
These fluorescein dyes were subsequently used together with MitoTracker DeepRed FM in colocalisation studies to verify the ability of the new vector to target the mitochondria, as well as to quantify the relative uptake of the new PPN⁺ vector. HeLa cells were treated the MitoTracker together with compound 6/7, and the images were examined under a confocal laser microscope (see ESI for experimental details). The fluorescence intensity ratio between the two fluorophores (Fluo/MT) are calculated to compare the relative accumulation of the two compounds. The confocal microscopy images and Fluo/MT values are presented in Figure 5 and Table 2 respectively. The images and Fluo/MT values evidently revealed that compound 7 had a much higher rate of accumulation. A high degree of colocalization was observed as well, indicating that 7 had localised within the mitochondria, as supported by the Van Steensel’s cross correlation function, high Pearson’s coefficient and Mander’s coefficients (see Table 2 and ESI). On the other hand, compound 6 had a low uptake with poorer colocalisation. This observation is consistent with other studies, where it was noted that an increase in the alkyl linker to 10 carbons was necessary for higher uptake for a similar fluorophore. The Fluo/MT was approximately 10-fold higher in 7, showing drastically improved performance of the PPN⁺ moiety in mitochondrial targeting compared to the traditional TPP⁺.

Conclusion

In summary, we designed and synthesised aminophosphonium and phosphazenylphosphonium main group frameworks as enhanced mitochondrial targeting vectors. We have also demonstrated the first application of PPN⁺ compounds as viable mitochondrial delivery vectors through confocal imaging. These species were shown to be superior molecular vectors compared to the current ‘gold standard’, with a 10-fold increase in mitochondrial accumulation while maintaining high mitochondrial selectivity.

Efforts were made to find new molecular parameters to adequately explain trends observed in cytotoxicity and lipophilicity, with the maximum of the molecular electrostatic potential surface of the cations, \( V_{max} \), showing good correlation for the newly species synthesised. This new parameter demonstrates the positive effect of charge redlocalisation on mitochondrial accumulation.

Benchmarking against a wider range of molecular vectors in the future, may further broaden the applicability of the parameters discussed to other subsets of compounds. Thus, there is a need to study and expand the scope of modified TPP⁺ compounds as well as alternative delivery vectors – such as the ones described herein in the search for a universal mitochondrial accumulation predictive tool.

This work expands the scope of mitochondrial delivery vectors based on main group frameworks and underscores the need to explore non-conventional delivery vectors beyond the traditional TPP⁺ systems toward future enhanced mitochondrial therapies.

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Keywords: Mitochondria • Main Group • TPP⁺ • DFT • Imaging

Triphenylphosphonium (TPP⁺) moieties represent the “gold standard” for mitochondrial delivery. Herein, we explore aminophosphonium (PN⁺) and phosphazenylphosphonium (PPN⁺) frameworks as novel delivery vectors. We demonstrate the PPN⁺ molecular platform as a highly promising mitochondrial delivery vector due to both its high lipophilicity and unique electronic properties, as shown by density functional theory and molecular dynamics calculations.

Institute and/or researcher Twitter usernames: @FGarcia_Group