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Long chain amines and long chain ammonium salts as novel inhibitors of dynamin GTPase activity

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Abstract—We examined a number of ligands with the view of inhibiting the GTPase activity of dynamin. Dynamin contains a pleckstrin homology (PH) domain that interacts with lipids. We report a series of simple lipid-like molecules that display moderate inhibitory activity. Inhibitory activity is linked to chain length and quaternarization of the terminal amine. A change in the counterion, Cl versus Br or I, had little effect on potency. However, introduction of a hydrophobic collar proximal to the charged site was beneficial to dynamin GTPase inhibitory action. The most potent compound was myristoyl trimethyl ammonium bromide (MTMAB, IC_{50} 3.15 μM).

The uptake and recycling of extracellular material by mammalian cells proceeds via endocytosis.[1–3] Crucial to this activity is the formation of a myriad of different sized vesicles—from the large phagosomes and the smaller clathrin-coated vesicles to tiny synaptic vesicles. Endocytic mechanisms are subservient to a variety of cellular functions including the uptake of cellular nutrients, regulation of cell-surface receptor expression and signaling, antigen presentation and maintenance of synaptic transmission. Although there are a variety of endocytic pathways, two have been biochemically well characterized, rapid *synaptic vesicle endocytosis* (SVE) that follows vesicle exocytosis in nerve terminals. SVE serves to retrieve empty synaptic vesicles for later refilling.[4,5] The second is *receptor-mediated endocytosis* (RME) which is initiated upon ligand binding to cell surface receptors and occurs via clathrin-coated pits in all cells.[4] SVE and RME perform distinct functional roles and share the same underlying protein machinery, but they may use distinct isoforms of the same protein.

Alzheimer's disease, Huntington's disease, Stiff-person syndrome, Lewy body dementias, and Niemann-Pick type C disease are illustrative of human pathological conditions within which defects in endocytosis have been implicated.[6–9] Endocytic pathways are also utilized by viruses, toxins and symbiotic microorganisms to gain entry into cells.

Dynamin was the first of a new family of *dynamin-like* GTP-binding proteins that share similar GTPase domains. This domain is crucial for vesicle fission, and consequently represents an important step in the endocytic pathway.[4,10] There are three dynamin genes, with dynamin I in neurons, II being ubiquitously expressed and III in neurons and testes.[5,11] All dynamins have four main domains, which are potential drug targets.

The *GTPase domain* has an unusually low affinity for GTP (10–25 μM) and extremely high turnover rates compared with other GTPases. This activity is required for vesicle fission.[11,12] The crystal structure of this domain of dynamin from *Dictyostelium* was recently

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solved.[13] The globular structure contains the G-protein core fold, but the normal six-stranded β-sheet is extended to an eight-stranded one by a unique 55 amino acid insertion. The *pleckstrin homology* (PH) domain is both a targeting domain and potentially a GTPase inhibitory module and is essential for endocytosis.[5] Dynamin interacts with lipids via this domain, and dynamin binding to nanotubules containing phosphatidylinositol bisphosphate (PtdIns(4,5)P_2) greatly stimulates GTPase activity.[14] The PH domain is not needed for self-assembly or GTPase activity—simply deleting it (delta-PH dynamin) maximally increases intrinsic GTPase activity.[15] The crystal structure of this domain has also been solved, and is typical of a lipid-binding domain.[16,17] The *GTPase effector domain* (GED) controls dynamin–dynamin interactions and dynamin assembly into its basic tetrameric configuration, tetramers being subunits of the rings. About 28–32 tetramers cooperatively self-assemble as a single ring[18] or as a helix around PtdIns(4,5)P_2-containing lipid mixtures.[14] GED accounts for tetramer self-association by binding to the GTPase domain.[11] Mutations in GED affect endocytosis in cells, some decreasing and some (surprisingly) increasing endocytosis. GED acts like a GTPase activator protein to stimulate GTPase activity.[19] The *proline-rich domain* (PRD) at dynamin's C-terminus interacts with many SH3 domain-containing proteins[5,12] and calcineurin[20] and is the site for in vivo dynamin phosphorylation.[21]

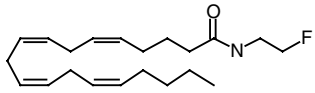
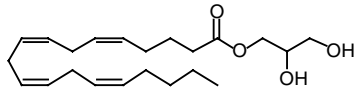
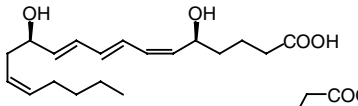
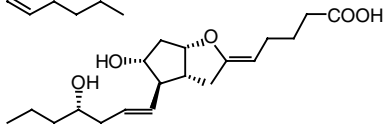
The nucleotide-free and GDP-bound structures of the GTPase domain are very similar, supporting that dynamin's major conformational GTP binding or hydrolysis remains essentially the same. Thus targeting the GTPase activity of dynamin is an attractive candidate for an endocytosis inhibitor. Multiple endocytosis inhibitors exist: cationic amphiphilic drugs, for example, chlorpromazine,[22] concanavalin A, phenylarsine oxide,[23] dansylcadaverine,[24] intracellular potassium depletion,[25] intracellular acidification,[26] and decreasing medium temperature.[12] Each has poor specificity and limited utility.

Herein we report our preliminary studies on the inhibition of dynamin GTPase activity as a prelude to the development of drugs that may inhibit endocytosis.

The PH domain of a protein is a relatively specific lipid binding domain, consequently our initial investigations revolved around primary screening of a number of lipid-like molecules, followed by biological evaluation.

Our initial screening protocol, at all times utilizing pure dynamin I GTPase[27] allowed primary screening at ca. 300 μM drug concentration, whilst removing any possibility of cellular based interferences giving rise to false positives. Those compounds showing >70% inhibition at this level were then subjected to full dose–response analysis. The outcomes of this primary screening are shown in Table 1, again with pure dynamin I GTPase. We reasoned that as the PH domain is responsible for binding lipids, hydrophobic molecules would potentially inhibit dynamin activity. Thus we examined a number of readily available fatty acids. As can be seen from Table 1, the more complex examples tested were poor inhibitors of dynamin GTPase activity. Interestingly both the arachindonyl glycerol and leukotriene B_4 (Table 1, entries 1 and 3) increased dynamin GTPase activity at a variety of concentrations. Of greater interest was prostaglandin I_2 showing 45% inhibition at 300 μM (Table 1, entry 4). We subsequently screened the less complex myristic acid and found no improvement in inhibitory activity (entry 5). However, myristylamine showed considerable improvement in potency (Table 1, entry 6, $IC_{50} = 33.5 \pm 0.71$ μM). Given the observed potency of this simple myristoylated analogue we then sought to examine the effect of chain length on dynamin GTPase inhibition.

Table 1. Inhibition of dynamin I GTPase activity by long chain acids and amines

Entry	Compound	IC_{50} (μM)
1		>300
2		70%[a]
3		>300[b]
4		45%[c]
5	$CH_3(CH_2)_{12}COOH$	>100
6	$CH_3(CH_2)_8CH_2NH_2$	33.5 ± 0.71
7	$CH_3(CH_2)_{10}CH_2NH_2$	12.9 ± 2.40
8	$CH_3(CH_2)_{12}CH_2NH_2$	20.25 ± 9.40
9	$CH_3(CH_2)_{16}CH_2NH_2$	28.15 ± 6.72
10	$CH_3(CH_2)_{18}CH_2NH_2$	>100
11	$NH_2CH_2(CH_2)_{10}CH_2NH_2$	39.65 ± 5.59

[a] At 3 μM; 30 μM had no effect and 300 μM increased dynamin GTPase activity.
[b] Increased dynamin GTPase activity at 30 μM.
[c] At 300 μM.

Given the lipid binding nature of the PH domain we rationalized that such modifications would have an effect on both drug solubility and in binding affinity. Alkyl chain length modification had little effect between C10 and C18 with these analogues being essentially equipotent (Table 1, entries 6–9, IC_{50} ca, 30 ± 10 μM), however extension beyond C18 effectively rendered these amines inactive (Table 1, entry 10, IC_{50} >100 μM). Returning to a shorter alkyl chain, for example the simpler 1,12-diaminododecane, reinstated the previously observed level of inhibition (Table 1, entry 11, $IC_{50} = 39.65 \pm 5.59$ μM).

In an attempt to improve the water solubility of the key analogues, we next examined a series of simple ammonium salts. The results of biological screening are shown in Table 2.

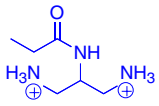
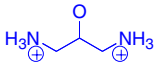
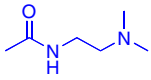
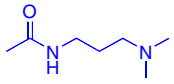
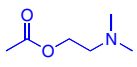
Table 2. Inhibition of dynamin I GTPase activity by long chain ammonium salts

Entry	Compound (alkyl chain — head group — counter ion)			IC_{50} (μM)
	Alkyl Chain	Head Group	Counter Ion	
1	$CH_3(CH_2)_{10}CH_2$	NH_3^+	Cl^-	18.24 ± 0.12
2	$CH_3(CH_2)_{12}CH_2$	NH_3^+	Cl^-	8.74 ± 0.01
3	$CH_3(CH_2)_{12}CH_2$	NH_3^+	Br^-	14.93 ± 0.19
4	$CH_3(CH_2)_{16}CH_2$	NH_3^+	Cl^-	31.07 ± 9.36
5	$CH_3(CH_2)_{16}CH_2$	NH_3^+	Br^-	>100
6	$CH_3(CH_2)_{10}CH_2$	$N^+(CH_3)_3$	Br^-	16.32 ± 1.02
7	$CH_3(CH_2)_{12}CH_2$	$N^+(CH_3)_3$	Cl^-	9.68 ± 2.38
8	$CH_3(CH_2)_{12}CH_2$	$N^+(CH_3)_3$	Br^-	5.79 ± 2.06
9	$CH_3(CH_2)_{12}CH_2$	$N^+(CH_3)_3$	I^-	8.15 ± 7.42
10	$CH_3(CH_2)_{14}CH_2$	$N^+(CH_3)_3$	Br^-	4.37 ± 0.60
11	$CH_3(CH_2)_{16}CH_2$	$N^+(CH_3)_3$	Br^-	3.15 ± 0.64

In all instances the simple ammonium salts afforded a modest improvement in inhibition over the corresponding free amine. This is evident on comparison of myristylamine (Table 1, entry 8, $IC_{50} = 20.25 \pm 9.40$ μM) with myristoylated ammonium chloride and bromide (Table 2, entries 2 and 3, $IC_{50} = 8.74 \pm 0.01$ and 14.93 ± 0.19 μM, respectively). The effect of counterion was minimal. More interesting is the effect of methylation of the free amine, with the trimethyl ammonium salts being uniformly more potent than either free amine or the simple HCl or HBr salts (Table 2, entries 7–11). Again the effect of counter ion is minimal (Table 2, entries 7 (Cl) $IC_{50} = 9.68 \pm 2.38$; 8 (Br) $IC_{50} = 5.79 \pm 2.06$ and 9 (I) $IC_{50} = 8.15 \pm 7.42$ μM). However, examination of the octadecyl ammonium salts shows a different inhibitory profile, one in which the nature of the counterion had a pronounced effect. Modest inhibition is noted for the HCl salt (Table 2, entry 4, $IC_{50} = 31.07 \pm 9.36$ μM), dropping off with the HBr salt (Table 2, entry 5, $IC_{50} > 100$ μM), and significantly improving with the trimethyl ammonium bromide salt (Table 2, entry 11, $IC_{50} = 3.15 \pm 0.64$ μM).

Given the consistent improvement in dynamin GTPase inhibition after the introduction of a small alkyl group at the ammonium site, we next sought to examine the effect of alkyl group modification at this site. A series of simple synthetic manipulations rapidly afforded analogues of the type shown in Table 3. As can be seen, the introduction of a collar had no detrimental effect, even

Table 3. Inhibition of dynamin I by long chain ammonium salts

Entry	Compound (alkyl chain — head group — counter ion)			IC_{50} (μM)
	Alkyl Chain	Head Group	Counter Ion	
1	$CH_3(CH_2)_{10}CH_2$	$N(CH_3)_2$	–	14.79 ± 2.04
2	$CH_3(CH_2)_{10}CH_2$	$NH^+(CH_3)_2$	$Cl^\ominus$	33.8 ± 0.99
3	$CH_3(CH_2)_{10}CH_2$	$NH^+(CH_3)_2$	$Br^\ominus$	30 ± 2.79
4	$CH_3(CH_2)_{10}CH_2$	$N^+(CH_3)_2CH_2CH_2CH_3$	$Br^\ominus$	10.14 ± 0.11
5	$CH_3(CH_2)_{12}CH_2$	$N^+(CH_3)_2CH_2CH_2CH_2CH_3$	$Br^\ominus$	8.85 ± 0.18
6	$CH_3(CH_2)_{12}CH_2$	$N^+(CH_3)_2CH_2CH_2CH_2CH_2CH_3$	$Br^\ominus$	8.02 ± 1.99
7	$CH_3(CH_2)_{12}CH_2$	$N^+(CH_3)_2CH_2CH_2OH$	$Br^\ominus$	9.36 ± 0.93
8	$CH_3(CH_2)_{12}CH_2$	$N^+(CH_3)_2CH_2Ph$	$Br^\ominus$	19.61 ± 1.43
9	$CH_3(CH_2)_{12}CH_2$	$N^+(CH_2CH_3)_3$	$Br^\ominus$	8.45 ± 1.85
10	$CH_3(CH_2)_{14}CH_2$	$N^+(CH_2CH_3)_3$	$Br^\ominus$	9.62 ± 1.29
11	$CH_3(CH_2)_{14}CH_2$	$P^+(CH_2CH_2CH_2CH_3)_3$	$Br^\ominus$	10.19 ± 1.58
12	$CH_3(CH_2)_{10}CH_2$		$2\times Br^\ominus$	10.16 ± 0.17
13	$CH_3(CH_2)_{12}CH_2$		$2\times Cl^\ominus$	7.37 ± 1.97
14	$CH_3(CH_2)_{10}CH_2$		—	8.85 ± 2.19
15	$CH_3(CH_2)_{10}CH_2$		—	7.75 ± 0.27
16	$CH_3(CH_2)_{10}CH_2$		—	23.02 ± 4.27

in the absence of the ammonium salt, on the activity of simple analogues (cf. Table 3, entries 1–15, $IC_{50} = 7.37 \pm 1.97 - 33.8 \pm 0.99\,\mu M$). We also note that a change from ammonium to phosphonium elicited no change in inhibitory potential (Table 3, entry 11, $IC_{50} = 10.19 \pm 1.58\,\mu M$).

Conclusions

We have discovered a series of simple amines and ammonium salts, the most potent being myristoyl trimethyl ammonium bromide (MTMAB), displaying considerable promise as leads for the development of more potent analogues. Small molecule dynamin inhibitors have the potential to moderate the endocytic pathway in cells, and consequently may ultimately prove to have a significant impact on a wide range of human conditions. We will report the further development of this class of compounds in due course.

Acknowledgements

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