Neural actions of immunophilin ligands

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Immunophilins, protein receptors for immunosuppressant drugs such as cyclosporin A and FK506, are enriched far more in the brain than in the immune system. Drug–immunophilin complexes bind to calcineurin, inhibiting its phosphatase activity and leading to immunosuppressant effects. The immunophilin FKBP-12 (FK506 binding protein, 12 kDa) forms a complex with the ryanodine and inositol (1,4,5) trisphosphate (IP_3) receptors to regulate their physiological release of intracellular Ca^{2+}. Here, **Solomon Snyder and colleagues** describe how non-immunosuppressant as well as immunosuppressant immunophilin ligands are neurotrophic for numerous classes of damaged neurones, both in culture systems and intact animals. Their ability to stimulate functional regrowth of damaged sciatic, cortical cholinergic, dopamine and 5-HT neurones may have therapeutic relevance.

Immunophilins are the receptor proteins for the major immunosuppressant drugs cyclosporin A, FK506 and rapamycin. The majority of research on the immunophilins and their ligands has focused on cells of the immune system, especially lymphocytes, but the finding that the immunophilins are much more abundant in the nervous system than in the immune system led to research that reveals important roles for the immunophilins in multiple areas of neural function. These include regulation of nitric oxide (NO) neurotoxicity, neurotransmitter release, intracellular Ca^{2+} flux via the ryanodine and the inositol (1,4,5) trisphosphate (IP_3) receptors, as well as neurotrophic influences with therapeutic potential.

The immunosuppressants permit organ transplantation without the toxicity associated with earlier drugs that acted as general antimitotic agents[1]. Cyclosporin A (CsA) is an 11-member cyclic peptide, while FK506 and rapamycin are macrolide antibiotics. CsA and FK506 inhibit early, Ca^{2+}-dependent steps of the T-cell response to antigen and, thus, prevent the synthesis and secretion of interleukin 2 (IL-2). The similar pharmacological profile of these drugs led to the assumption that both act via similar molecular mechanisms. By contrast, rapamycin inhibits a later stage of the T-cell response to antigen, preventing IL-2-induced clonal proliferation of T cells by blocking signalling through the IL-2 receptor. Because of its pharmacological profile, one would expect rapamycin to act via a different molecular mechanism than CsA and FK506.

The first approach to understanding the molecular actions of the immunosuppressants involved the identification of receptor binding sites. Handschumacher and colleagues[2] discovered cyclophilin, an 18 kD soluble protein that binds CsA with high affinity. Subsequently, a family of cyclophilins has been identified[3,4] with varying molecular weights and subcellular locations. Surprisingly, FK506 does not bind to any of the cyclophilins but instead binds selectively and with high affinity to another small soluble protein, FKBP-12, which is also part of a large family[4,5].

Although there is essentially no amino acid sequence homology between FKBP-12 and any of the cyclophilins, they do share one commonality. Both possess peptide-prolyl isomerase or rotamase activity[3]. Peptide bonds in proteins exist in *cis* and *trans* isomers because of their partial double-bond character. Though this rotamase activity affects the conformation of a few known proteins, the immunophilins may primarily serve as scaffold or chaperone proteins with rotamase actions facilitating protein–protein binding (see below). Ligands of the immunophilins inhibit their rotamase activity, and this finding was initially suggested to be a mechanism for their immunosuppressant effects. However, numerous compounds that bind to the immunophilins and inhibit their rotamase activity lack immunosuppressant actions[6]. Moreover, immunosuppressant drugs often act at low nanomolar concentrations, whereas tissue levels of some of the immunophilins are almost micromolar so that in intact tissues only a tiny percent of rotamase activity would be inhibited. Conceivably, immunosuppressants could act by binding to an immunophilin whose tissue density is extremely low. Alternatively, the drug–immunophilin complex might acquire a 'gain of function' and bind to a second protein with which neither drug nor receptor interacts alone.

Liu *et al.*[7] and Friedman and Weissman[8] identified the first drug–immunophilin target by showing that complexes of CsA–cyclophilin A and FK506–FKBP-12 interact with calcineurin, a Ca^{2+}–calmodulin activated protein phosphatase. One of the calcineurin substrates is the phosphorylated form of the transcription factor nuclear factor of activated T cells (NF-AT), which activates the transcription of many T-cell specific genes including those for IL-2 and its receptor. To enter the nucleus and influence IL-2 transcription, NF-AT must be dephosphorylated. Binding of the drug–immunophilin complex to calcineurin inhibits its phosphatase activity, leading to elevated levels of phosphorylated NF-AT which cannot enter the nucleus to activate gene transcription (Fig. 1). The relative potencies of numerous drugs in inhibiting calcineurin correlate closely with their immunosuppressant potencies, establishing that CsA, FK506 and related drugs act by inhibiting calcineurin and decreasing IL-2 formation[6].

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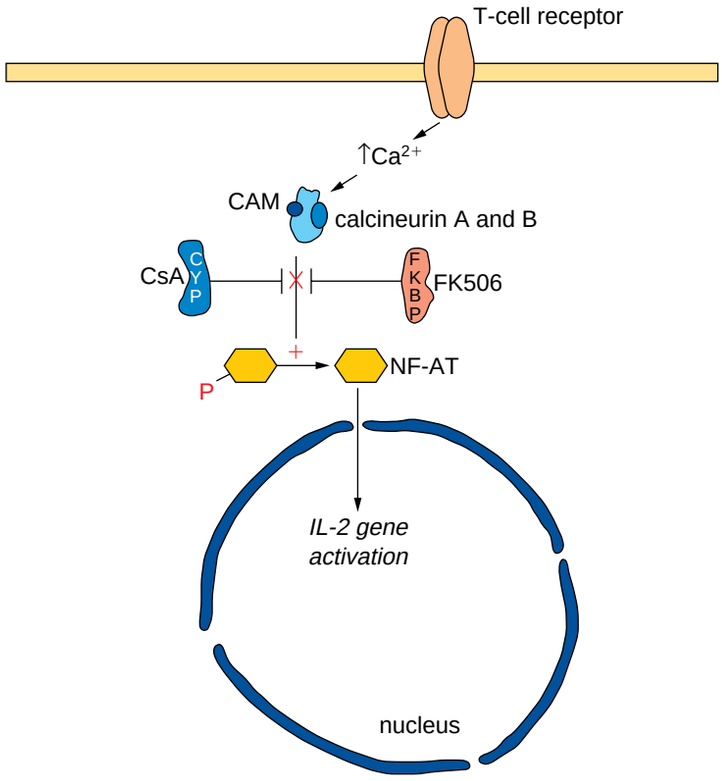

Fig. 1. Inhibition of T-cell activation by cyclosporin A (CsA) and FK506. Foreign antigens displayed on the surface of antigen-presenting cells activate the T-cell receptor to initiate signalling pathways that lead to increases in intracellular Ca^{2+}. Ca^{2+} binds to calmodulin (CAM) and calcineurin B, which activate the phosphatase activity of the catalytic subunit (calcineurin A) of calcineurin. The phosphatase dephosphorylates the nuclear factor of activated T-cell (NF-AT), allowing it to enter the cell nucleus and activate the transcription of several T-cell-specific genes, including those for interleukin 2 (IL-2) and the IL-2 receptor. The CsA–cyclophilin A (CyP) and FK506–FKBP-12 (FKBP) complexes bind to and inhibit calcineurin, preventing NF-AT dephosphorylation and its subsequent translocation to the nucleus. Activation of the IL-2 gene leads to the synthesis and secretion of IL-2 by the activated T-cell clone.

Though rapamycin binds with high affinity to FKBP-12, the drug–immunophilin complex does not bind to calcineurin but instead to a protein designated rapamycin and FKBP-12 target-1 (RAFT1)[9], FKBP and rapamycin associated protein (FRAP)[10] or mammalian target of rapamycin (TOR)[11–13]. RAFT1 phosphorylates the protein translation regulator 4E-BP1 (Ref. 14).

Neural roles of immunophilins: NO, neurotoxicity and neurotransmitter release

The surprising finding that levels of FKBP-12 in the brain are up to 50 times greater than those in tissues of the immune system provided the first suggestion for a neural role of the immunophilins[15]. Both FKBP-12 and cyclophilin are almost exclusively neuronal throughout the brain with marked regional variations that closely resemble the distribution of calcineurin[16]. Particularly high densities occur in granule cells of the cerebellum, the major layers of the hippocampus and a striatonigral pathway. The co-localizations of calcineurin with FKBP-12 and cyclophilin provided the first suggestion that calcineurin might interact physiologically with the immunophilins. To identify calcineurin substrates influenced by immunosuppressant drugs, proteins that showed an enhanced level of phosphorylation when incubated in the presence of FK506 were screened for in brain homogenates. Two such proteins were found, GAP-43, which mediates neuronal process elongation, and neuronal nitric oxide synthase (nNOS)[15,17].

Phosphorylation of nNOS by protein kinase C markedly inhibits its catalytic activity[18]. The neurotoxic effects of glutamate acting through *N*-methyl-D-aspartate (NMDA) receptors involve NO, as NOS inhibitors[19] and nNOS gene knockout[20] block such toxicity. Low concentrations of FK506 also block NMDA neurotoxicity[17] (Fig. 2) and, as expected, rapamycin acts as an antagonist and prevents the neuroprotective actions of FK506. By inhibiting calcineurin, FK506 increases phosphorylated levels of nNOS, reducing its catalytic activity and providing neuroprotection similar to the effects of NOS inhibitors. Like NOS inhibitors, FK506 also prevents neural damage associated with vascular stroke[21].

Immunosuppressant inhibition of calcineurin also modulates neurotransmitter release with the specific effect dependent on which calcineurin substrate is affected. FK506 potently prevents spontaneous as well as K^+ depolarization-induced release of neurotransmitters from PC12 cells, and these effects are blocked by rapamycin[22]. FK506 also blocks NMDA-induced release of glutamate from brain synaptosomes. By contrast, the release of several neurotransmitters from synaptosomes following K^+ is greatly enhanced by low nanomolar concentrations of CsA and FK520, a close analogue of FK506 (Ref. 22). These two immunophilin ligands also augment glutamate release from synaptosomes treated with K^+ channel blockers[23].

Such divergent and apparently contradictory effects of immunophilin ligands might be explained by the inhibition of calcineurin and subsequent effects on its diverse collection of substrates. NO is required for neurotransmitter release in PC12 cells and NMDA-stimulated synaptosomes, as NOS inhibitors block release from these systems[24]. By inhibiting calcineurin-mediated dephosphorylation of nNOS, immunosuppressant drugs enhance the phosphorylation of nNOS and reduce its catalytic activity, leading to block of transmitter release (Fig. 2). In other systems, such as synaptosomes treated with K^+ channel blockers, stimulation of transmitter release may be associated with increased phosphorylation (via calcineurin inhibition) of proteins whose phosphorylated state promotes neurotransmitter release. Examples of such proteins include synapsin I (Ref. 22) and dynamin I (Ref. 23). Synapsin I is a prominent synaptic vesicle-associated protein, while dynamin I is a GTPase whose catalytic activity is enhanced by phosphorylation leading to augmented recycling of synaptic vesicles and increased neurotransmitter release.

Immunophilins regulate intracellular Ca^{2+} release

Since the initial identification of FKBP-12 as the major receptor protein for FK506, several proteins have been

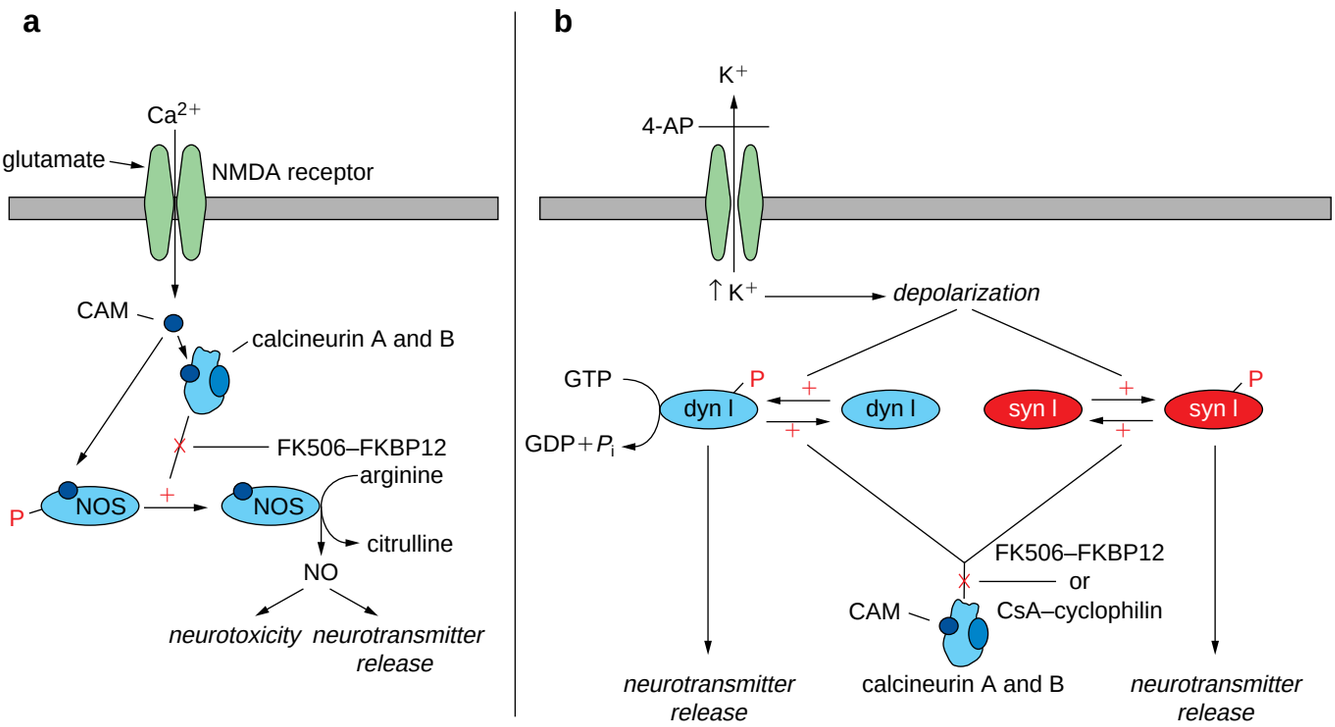

Fig. 2. Immunophilin regulation of neurotransmitter release and neurotoxicity. **a:** Regulation via nitric oxide synthase (NOS). Neurotransmitter release in PC12 cells depends on NOS activity. In its unphosphorylated form, NOS is active, and it is inactivated by protein kinase C (PKC) phosphorylation. Calcineurin returns NOS to its active state by dephosphorylation. The immunophilin ligands such as FK506 inhibit calcineurin, keeping the NOS in its inactive state which decreases neurotransmitter release. Production of NO by NOS also mediates glutamate excitotoxicity. Calcineurin inhibition by FK506 prevents dephosphorylation of NOS, allowing NOS to remain in the inactive state. The diminished NO production accounts for the neuroprotective effect of immunophilin ligands. **b:** Regulation via dynamin I and synapsin I. Dynamin I (dyn I) and synapsin I (syn I) contribute to neurotransmitter release. Both proteins are active in the phosphorylated state, and calcineurin dephosphorylation leads to their inactivation. By inhibiting calcineurin, cyclosporin A (CsA) and FK506 allow dynamin I and synapsin I to remain active, thereby enhancing neurotransmitter release.

found to associate with FKBP-12 via the FK506 binding site. The yeast two-hybrid technique identified type I receptor for transforming growth factor-β (TGF-β)[25] and a novel protein FKBP-associated protein-48 (FAP48)[26]. Of the two forms of the TGF-β receptor, type II, when bound to TGF-β, phosphorylates type I on serine–threonine to activate the receptor. Binding of FKBP-12 to the type I receptor interrupts TGF-β signalling[25]. Downstream, TGF-β regulates extracellular matrix dynamics in neurones as well as other tissue.

Biochemical purification led to the identification of two more FKBP-12-associated proteins with related functions, the ryanodine receptor and the inositol (1,4,5) trisphosphate (IP_3) receptor. Both the ryanodine receptor and IP_3 receptor mediate intracellular Ca^{2+} release. Purification of the ryanodine receptor leads to copurification of FKBP-12 (Refs 27, 28). In contrast to the situation with calcineurin, treatment of the complex with FK506 dissociates FKBP-12 from the ryanodine receptor (Fig. 3), suggesting that the FKBP-12–ryanodine receptor association involves the FK506 binding pocket. Loss of FKBP-12 renders the ryanodine receptor 'leaky' to Ca^{2+} (Ref. 29). FKBP-12 is also tightly bound to highly purified preparations of the IP_3 receptor and dissociation of FKBP-12 from the IP_3 receptor by FK506 causes leakiness of the IP_3 receptor Ca^{2+} channel[30] (Fig. 3).

Calcineurin is also part of the IP_3 receptor–FKBP-12 and RyR–FKBP-12 receptor complexes and FK506 or rapamycin disrupts the interaction[31]. In the IP_3 receptor complex calcineurin regulates phosphorylation of the IP_3 receptor especially by protein kinase C, which might conceivably be another member of the complex. Presumably, this macromolecular complex facilitates the cycle of receptor phosphorylation and dephosphorylation which may participate in the generation of intracellular Ca^{2+} oscillations. FKBP-12 appears to act as a scaffold holding the other proteins together and is analogous to the A kinase anchoring protein, AKAP79 which brings together protein kinase A and calcineurin in a molecular complex[32]. Utilizing the yeast two-hybrid technique we demonstrated that FKBP-12 binds the IP_3 receptor at residues 1400–1401, a leucyl-prolyl dipeptide that structurally resembles FK506 (Ref. 33). Binding at this site anchors calcineurin to the IP_3 receptor. Binding at the leucyl-prolyl site would suggest that FKBP-12 exerts rotamase activity to fold the IP_3 receptor. However, derivatives of FKBP-12 devoid of rotamase activity serve as scaffolds for the binding complex just as well as wild-type FKBP-12 (Ref. 33).

Immunophilins mediate nerve growth

On the basis of observations of high levels of FKBP-12 in peripheral nerves and growth cones of neonatal

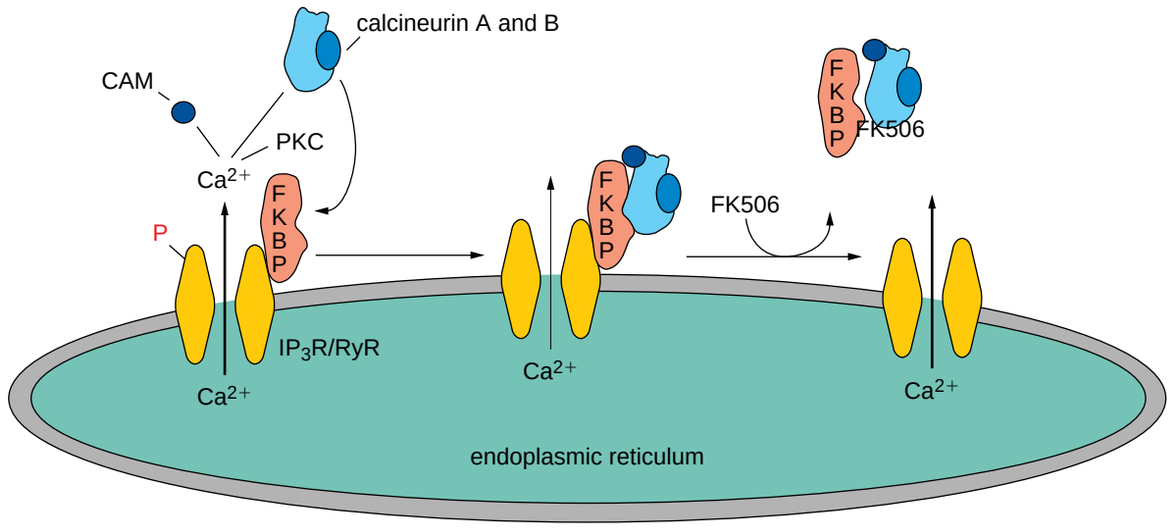

Fig. 3. Calcineurin associates in a Ca^{2+}-sensitive manner with the inositol (1,4,5) trisphosphate receptor (IP_3R) via an FKBP-12 anchor. The IP_3 receptor is an integral membrane protein of the endoplasmic reticulum and is shown complexed with FKBP-12. When the receptor is activated by IP_3, Ca^{2+} is released from the lumen of the endoplasmic reticulum via the IP_3 receptor channel and results in increases in local Ca^{2+} concentrations. This increase activates protein kinase C (PKC), which phosphorylates the IP_3 receptor and further increases IP_3-mediated Ca^{2+} flux (shown as a thick arrow). Increased Ca^{2+} levels also activate calmodulin (CAM) and calcineurin (CN), causing an association of CN with the IP_3 receptor–FKBP-12 complex via FKBP-12 and activation of the phosphatase activity of calcineurin. The activated CN dephosphorylates the PKC phosphorylation site on the IP_3 receptor and decreases Ca^{2+} flux (thin arrow). The addition of FK506 displaces FKBP-12 and calcineurin from the receptor and results in suboptimal subunit cooperativity and leaky Ca^{2+} channels (thick arrow). RyR, ryanodine receptor.

neurones[15] as well as the dramatic upregulation of FKBP-12 and growth-associated protein 43 kD (GAP-43) following damage to the sciatic or facial nerves[34] potential neurotrophic effects of immunophilins were investigated. Striking neurotrophic effects of FK506 were observed in PC12 cells and sensory ganglia with subnanomolar potencies[35], and these effects were also observed with rapamycin and CsA. In intact animals, Gold *et al.*[36,37] reported enhanced regrowth and functional recovery of crushed sciatic nerves following FK506 treatment. More recently, it was shown that non-calcineurin binding analogues of FK506 and cyclosporin also promote neurite outgrowth. For example, L685818, which differs only modestly in structure from FK506, is as effective as FK506 in stimulating regeneration and functional recovery following sciatic nerve crush[38].

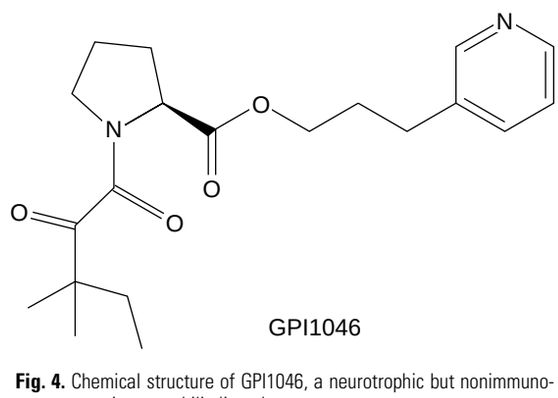

Fig. 4. Chemical structure of GPI1046, a neurotrophic but nonimmunosuppressant immunophilin ligand.

These immunosuppressive derivatives bind to the immunophilins with similar potencies as the parent drugs, but the drug–immunophilin complex fails to bind to and inhibit calcineurin and these derivatives are not immunosuppressants. As the non-immunosuppressant derivatives are just as neurotrophic as the parent drugs, the immunosuppressive properties of FK506 and cyclosporin could be functionally dissociated from their neurotrophic effects[38].

FK506 possesses separate domains that bind FKBP-12 and calcineurin, respectively. Using principles of structure-based drug design, novel compounds containing an FKBP-12 binding domain but lacking the ability to bind calcineurin were synthesized (Fig. 4). These compounds retain potent neurotrophic effects[39–41]. One of these agents, GPI1046, detectably augments neurite outgrowth of sensory ganglia at 1 pM, with maximal effects at 1 nM, comparable to the maximal actions of nerve growth factor. Low doses of GPI1046 also augment recovery of damaged sciatic nerves. While cross sectional area of the nerve is augmented by drug treatment, even more pronounced effects are noted in recovery of myelination which may be relevant for therapy of demyelinating diseases.

The neurotrophic effects of immunophilin ligands in culture systems resemble those of neurotrophic proteins such as nerve growth factor, brain-derived neurotrophic factor, neurotrophin (NT)-3 and NT-4 and are exerted at similar molar potencies. While the neurotrophic proteins each act on a selected repertoire of neuronal systems, immunophilin ligands are neurotrophic in every system that has been examined. In intact animals, drugs such as GPI1046 enhance the recovery of 5-HT-containing

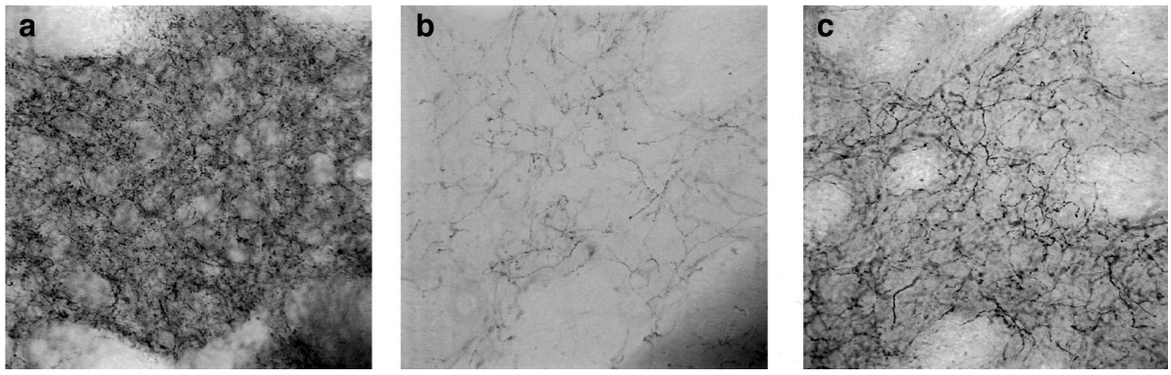

Fig. 5. Tyrosine hydroxylase staining, a marker for dopamine-containing neurones (**a;** Veh/Veh), increased following GPI1046 treatment of rats whose corpus striatum dopamine-containing neurones were lesioned with 6-hydroxydopamine (**b;** 6-OHDA/Veh). One week after 6-hydroxydopamine injections into the substantia nigra, GPI1046 (10 mg kg^{-1}, subcutaneous administration) was administered for five days (**c;** 6-OHDA/10 mg kg^{-1} GPI1046). Modified figure, reproduced with permission from Ref. 39.

neurones in the brain following lesions with *p*-chloroamphetamine[39] and of central cholinergic neurones following fimbria-fornix lesions[42].

Dopamine-containing neurones that have been lesioned with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or 6-hydroxydopamine[39], regrow following GPI1046 treatment (Fig. 5), and the regenerated dopamine-containing neurones are functional. Thus, following unilateral 6-hydroxydopamine lesions, rats rotate. In animals treated with GPI1046 a week after 6-hydroxydopamine lesion, rotations are abolished despite the fact that drug treatment has restored striatal dopamine only to about 30% of control levels[39]. This fits with abundant evidence that only about a third of normal dopamine innervation is required for physiological motor activity.

How do the immunophilin ligands exert their neurotrophic effects? In PC12 cells the drugs act only in the presence of nerve growth factor and augment nerve growth factor potency about tenfold[35]. In sensory ganglia neurotrophic effects do not require the addition of any neurotrophic protein, but glial cells in the ganglia can manufacture endogenous neurotrophic proteins. The neurotrophic actions of immunophilin ligands are restricted to damaged neurones, as studies have not yet been able to demonstrate neurite outgrowth in normal central or peripheral neurones, whereas neurotrophic proteins do elicit such outgrowth[40].

The therapeutic potential of neurotrophic drugs is considerable, with potential targets including diabetic neuropathy, amyotrophic lateral sclerosis, spinal cord injury, Parkinson's disease, Alzheimer's disease and stroke. Though neurotrophic proteins have been exploited for potential therapy in these conditions, delivering a protein to the CNS is difficult, and these proteins do stimulate nerve outgrowth in normal tissues, which could lead to adverse effects. By contrast, recently developed immunophilin ligands are small organic molecules which are orally effective, act only on damaged nerves and have ready access to the CNS (Ref. 39).

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Chemical name

GPI1046: 3-(3-pyridyl)-1-propyl(2s)-1-(3,3-dimethyl-1,2-dioxopentyl)-2-pyrrolidine carboxylate

Hydrophilicity/lipophilicity: relevance for the pharmacology and clinical effects of HMG-CoA reductase inhibitors

Bettina A. Hamelin and Jacques Turgeon

The recent development of specific competitive inhibitors of the hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase such as lovastatin, simvastatin, pravastatin and fluvastatin has provided an important new and effective approach to the treatment of hyperlipidaemia and atherosclerosis. These agents are designed to be hepatoselective because the primary site of cholesterol synthesis is the liver and peripheral inhibition of cholesterol synthesis would be more likely to cause adverse drug effects. In this review, **Bettina Hamelin and Jacques Turgeon** discuss how specific physico-chemical and pharmacological properties (first-pass effect or carrier-mediated uptake) confer hepatoselectivity to either lipophilic or hydrophilic HMG-CoA reductase inhibitors.

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The hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase catalyzes the conversion of hydroxymethylglutaryl (HMG) to mevalonate, an early and rate-limiting step in cholesterol synthesis[1]. Pharmacological inhibition of the HMG-CoA reductase causes a decrease in cholesterol synthesis which leads to upregulation of low-density lipoprotein (LDL) receptors, thus increasing the rate of removal of LDL from plasma.

HMG-CoA reductase is the target for a new class of highly effective inhibitors of cholesterol synthesis, namely lovastatin, simvastatin, pravastatin, fluvastatin and atorvastatin, the latter having been released in recent months[2–7]. The chemical structures of the first three HMG-CoA reductase inhibitors are closely related (Fig. 1) but the physico-chemical properties of lovastatin and simvastatin differ from those of pravastatin[8]. Fluvastatin represents the first entirely synthetic HMG-CoA reductase inhibitor and shares physico-chemical characteristics with pravastatin as well as with lovastatin and simvastatin (Fig. 1)[6]. On the other hand, the chiral Ca^{2+} salt of a pentasubstituted pyrrole, atorvastatin, was only recently synthesized and presents a distinct chemical structure[7]. Although this drug appears to be extremely effective, perhaps because its inhibition of the HMG-CoA reductase may be of longer duration compared to other agents of this class[7,9], little information on atorvastatin's pharmacological properties is currently available. Therefore, atorvastatin will not be considered here.

The objective of this review is to demonstrate, in a series of questions and answers, the necessity of particular pharmacological drug properties for appropriate structure/selectivity/activity relationships and their clinical relevance for therapeutic benefits and side-effect profiles of the currently most widely used HMG-CoA reductase inhibitors.

Question 1: What are the differences in physico-chemical properties of currently marketed HMG-CoA reductase inhibitors?

Lovastatin, simvastatin and pravastatin are structurally very similar (Fig. 1). In fact, lovastatin is derived from a fungal source and simvastatin and pravastatin are chemical modifications of lovastatin[2–4]. When the open hydroxy forms of these drugs are compared, lovastatin and simvastatin differ from pravastatin in that they possess a methyl instead of a hydroxyl moiety at position 6. Furthermore, simvastatin differs from the other two as it possesses an additional methyl group at position 2 on the butanoate lateral chain. While pravastatin is administered as the readily active open hydroxy-acid form, lovastatin and simvastatin are administered as inactive lactones (Fig. 1), which must be metabolized to their corresponding open hydroxy-acid forms in order to inhibit HMG-CoA reductase[2–4]. On the other hand, fluvastatin is the product of synthetic drug design and its structure is distinct from the described molecules (Fig. 1). However, it shares the dihydroxy heptanoic acid function with pravastatin and is, just like this latter agent, administered as an active β-hydroxy acid form[6]. Whether lactone prodrugs or active open hydroxy acids, all four HMG-CoA reductase inhibitors are provided with a bicyclic

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