An Alternative to Conventional Immunosuppression: Small-Molecule Inhibitors of Kv1.3 Channels

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A key step in the generation of an immune response, either physiological or pathophysiological (e.g., autoimmune), is the recognition of the antigen presented to the lymphocytes followed by their concomitant activation and clonal proliferation. The activation of T lymphocytes through the T-cell antigen receptor (TCR) initiates several downstream signaling pathways leading to the transcription of genes required for lymphocyte proliferation (e.g., interleukin-2 gene). One axis of downstream signaling is the phospholipase C-γ (PLCγ)–1,4,5-inositol trisphosphate (IP3)–Ca2+ pathway (1). IP3 releases Ca2+ from cytosolic Ca2+ stores, and this Ca2+ along with the Ca2+ influx through the plasma membrane generates a sustained elevation of the cytosolic free Ca2+ concentration, \([Ca^{2+}]_i\), required for efficient signal transduction. The calcium signal activates the Ca2+-calmodulin–dependent phosphatase calcineurin, which then dephosphorylates the transcription factor nuclear factor of activated T cells (NF-AT), thereby enabling it to accumulate in the nucleus and bind to the promoter element of the IL-2 gene (2). Conventional immunosuppressors, such as cyclosporine A and tacrolimus (FK506), interfere with lymphocyte activation by forming drug–immunophilin–calcineurin complexes, which inhibit the phosphatase activity of calcineurin, thereby preventing NF-AT translocation to the nucleus (3). These calcineurin...
inhibitors are leading compounds in the management of immune reactions during organ transplantation; however, their therapeutic use is associated with significant nephrotoxicity and an increased risk of infections and cancer(3).

The discovery (4) and the characterization of ion channels in T lymphocytes (5) recently opened a new opportunity for pharmacological interference with T cell activation (6, 7). The most abundant, as well as physiologically most important, voltage-gated K⁺ channel in human T lymphocytes, Kᵥ1.3, opens upon membrane depolarization with an activation threshold close to the resting potential of the cells. The Ca²⁺-activated potassium channel of human T cells, KᵥCa3.1 (formerly termed IKCa1), is activated solely by the rise of the cytosolic free calcium concentration to over ~200 nM, independently of the membrane potential. The calcium sensor is calmodulin that is permanently associated with the channel. These two types of K⁺ channels have similar structure; they are composed of four individual subunits, each subunit consisting of six transmembrane-spanning helices and the connecting intra- and extracellular loops. The extracellular loops connecting the fifth and sixth transmembrane helices from each subunit construct the extracellular vestibule of the channel, which serves as the receptor site for many blockers (see below), and also form the selectivity filter for K⁺ (8). Kᵥ1.3 and KᵥCa3.1 potassium channels have been identified in a variety of lymphocytes, and these channels along with the Ca²⁺ release–activated Ca²⁺ (CRAC) channel, the encoding genes of which have not been found yet (1, 7), regulate the Ca²⁺ influx through the plasma membrane. The membrane potential–independent CRAC channel is activated by the emptying of cytosolic Ca²⁺ stores by IP₃, and the electrical driving force for sustained Ca²⁺ entry is provided by the negative membrane potential maintained by the activity of Kᵥ1.3 and KᵥCa3.1 potassium channels (7). From the membrane potential–dependence of the Ca²⁺ signal one could predict that blockers of K⁺ channels can inhibit the proliferation of T cells, and therefore, suppress immune reactions. Accordingly, early studies reported that depolarization of T cells by specific blockers of Kᵥ1.3 was sufficient to inhibit human T cell activation in vitro (9). Other in vivo studies done over the last decade suggested that Kᵥ1.3 blockers caused generalized immunosuppression based on the ability of Kᵥ1.3 blockers [e.g., margatoxin, correolide, and kaliotoxin (Table 1)] to suppress delayed-type hypersensitivity (DTH) in mini-swine (10, 11). However, the cardinal issues in immunosuppression, namely, selective targeting of specific autoreactive responses without compromising essential immune functions and minimizing the side effects of the therapy, remained to be solved for K⁺ channel inhibitors.

Several recent findings have revolutionized the outlook for K⁺ channel inhibitors as potential immunosuppressors. One set of key observations described the specific expression level of Kᵥ1.3 channels in T lymphocyte subsets (5, 12), whereas structure-based drug design has resulted in the production of high-affinity and high-specificity blockers of Kᵥ1.3 channels (13).

All quiescent human T lymphocytes express ~200 to 400 Kᵥ1.3 channels and 8–10 KᵥCa3.1 channels per cell, so the membrane potential of these cells is controlled by Kᵥ1.3 channels. Upon activation by mitogens or antigens, the number of KᵥCa3.1 and Kᵥ1.3 channels increases differentially depending on the nature of the T cell subset (5). Naïve and central memory T cells (T_CM) (Box 1) transcriptionally increase the expression of KᵥCa3.1 channels; the number of channels increases up to ~500 per cell whereas the number of Kᵥ1.3 channels increases only modestly. Thus, these cells acquire a KᵥCa3.1 high/Kᵥ1.3 low phenotype. On the other hand, antigenic stimulation of effector memory T cells (T_EM) increases the number of Kᵥ1.3 channels dramatically (~1500 / cell) without any increase in the number of KᵥCa3.1 channels. The ion-channel phenotype of T_EM cells is therefore KᵥCa3.1 high/Kᵥ1.3 high. The difference in the ion-channel phenotype allows the proliferation of these cells to be modulated by Kᵥ1.3- or KᵥCa3.1-specific inhibitors. Activation of quiescent cells can primarily be inhibited by Kᵥ1.3 inhibitors, but not by KᵥCa3.1 blockers. Because the transcriptional upregulation of KᵥCa3.1 channels in naive and T_CM cells depends somewhat on the Ca²⁺ signal, the augmentation of the number of KᵥCa3.1 channels continues even if the initial activation of these cells is inhibited by Kᵥ1.3 channel blockers. Thus, control of the membrane potential is taken over by KᵥCa3.1 channels in activated naive and T_CM cells (both of which are KᵥCa3.1Kᵥ1.3 high phenotypes) and subsequent proliferation of these T cell subsets escapes further Kᵥ1.3 channel inhibition and becomes sensitive to KᵥCa3.1 channel blockade. The proliferation of activated T_EM cells, however, continues to be sensitive to Kᵥ1.3 blockade, because these cells express large amounts of Kᵥ1.3 channels. The use of selective Kᵥ1.3 inhibitors therefore allows the inhibition of the proliferation of T_EM cells, with minimal interference with the activation of naive and T_CM cells.

What is the significance of these findings? Beeton and colleagues report that in experimental autoimmune encephalomyelitis (EAE), which is a rat model for the human disease multiple sclerosis (MS), chronically activated encephalogenic T cells display an unusual channel phenotype. These repeatedly activated myelin basic protein–specific rat T cells (i.e., their TCRs specifically rec-

Table 1. Inhibitory Constants of Kᵥ1.3 Channel Blockers

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>EC₅₀ (nM)</th>
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<tbody>
<tr>
<td>Charybdotoxin</td>
<td>3</td>
</tr>
<tr>
<td>Margatoxin</td>
<td>110</td>
</tr>
<tr>
<td>Kaliotoxin</td>
<td>650</td>
</tr>
<tr>
<td>Pi2</td>
<td>44</td>
</tr>
<tr>
<td>ShK</td>
<td>11</td>
</tr>
<tr>
<td>Shk-Dap22</td>
<td>23</td>
</tr>
<tr>
<td>Correolide</td>
<td>90</td>
</tr>
<tr>
<td>Psora-4</td>
<td>2.9</td>
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*Measurements derived from references cited in this Viewpoint article.*
Box 1. Characteristics of Some T Lymphocyte Subsets

Naïve T cells are mature T cells that have not yet encountered an antigen. Based on the classification of T cells by the expression of the chemokine receptor CCR7 and the phosphatase CD45RA, these cells are of the CCR7+CD45RA+ phenotype. Following the encounter with an antigen, T cells divide and differentiate. Most of the progeny become short-lived armed effector cells, and some become long-lived memory cells. One type of memory cell is referred to as the effector memory cell (Tem) (CCR7−CD45RA−). This cell type can directly move to the sites of inflammation to promote effector functions along with the secretion of interferon-γ and tumor necrosis factor-α. The other type of memory cell is the central memory cell (Tcm) (CCR7+CD45RA−). Tcm migrate to the lymph node before moving to the site of inflammation, and they take longer to differentiate into effector cells and do not secrete as much cytokine.

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The Naïve T cells (Tem) are characterized by a high-affinity blocker for K_{Ca}v1.3 (K_{Ca}v1.3−/−) channels and a very limited expression of this channel in the blood cells. Endothelial cells can be inhibited by a K_{Ca}v1.3 blocker (TRAM-34) (5). Similar to other ion channels, the blockers of K_{Ca}v1.3 are isolated from the sea anemone Stichodactyla helianthus. In vitro experiments also demonstrate, that the symptoms of EAE could be greatly ameliorated by the application of the K_{Ca}v1.3−/− blocker Shk-Dap22 alone or in combination with a K_{Ca}v3.1 blocker (TRAM-34). Very recently, Chandy and colleagues have found that myelin-reactive Tem cells isolated from patients suffering from MS selectively acquire the K_{Ca}v3.1−/−K_{Ca}v1.3−/− channel phenotype, whereas T cells specific for control antigens express the naive and Tem phenotype (K_{Ca}v3.1highK_{Ca}v1.3low) (5). Moreover, the proliferation of K_{Ca}v1.3high Tem cells is completely and persistently inhibited by Shk toxin (Table 1), without affecting naive or Tcm lymphocytes. Thus, a K_{Ca}v1.3-based therapy that suppresses the activation of Tem cells without significant impairment of the proliferation of naive and Tcm cells might have use in the management of MS and other T cell–mediated immune diseases, such as type 1 diabetes mellitus (15), and also in chronic graft rejection and graft-versus-host disease sustained by chronically activated Tem cells (16). In addition to T lymphocyte subset–specific expression of K_{Ca}v1.3, the very limited expression of this channel (6, 7) in other tissues make K_{Ca}v1.3 an excellent target for specific immune suppression (17).

Parallel to the recent progress in understanding the significance of restricted expression of K_{Ca}v1.3, extensive research has been performed in several laboratories in order to find specific, high-affinity blockers for K_{Ca}v1.3 channels. Similar to other ion channels’ inhibitors, the blockers of K_{Ca}v1.3 were isolated from animals, mostly from the venom of scorpions [reviewed in (18)]. These peptide toxins are made up of approximately thirty-five amino acids, and their rigid and conserved three-dimensional structure is provided by three or four disulfide bridges. The binding site of these toxins is generally in the pore of the channel, bound toxins plug the ion conduction pathway resulting in the block of the ionic currents. The most potent natural toxin blockers of K_{Ca}v1.3 channels are characterized by equilibrium dissociation constants in the low nM and pM range. These include Pi2 toxin from Pandinus imperator (44 pM (19) and Table 1) and many others reviewed recently (6, 17). The most potent blocker of K_{Ca}v1.3, Shk, has a dissociation constant for K_{Ca}v1.3 of −11 pM (14). Based on the molecular models of the K_{Ca}v1.3 and K_{Ca}v3.1 pore and the crystal structure of KcsA channel (8), highly selective peptide inhibitors of these channels were synthesized recently. Guided by some unique features of the K_{Ca}v1.3 pore, the structure of the Shk toxin, and the careful analysis of interacting residues between Shk and the pore of K_{Ca}v1.3, a highly selective K_{Ca}v1.3 inhibitor was generated by replacing the central Lys22 in the Shk with a shorter, positively charged non-natural amino acid: diaminopropionic acid (14). The resulting peptide blocker, Shk-Dap22, retained high affinity for K_{Ca}v1.3 (Kd = 23 pM vs 11 pM for the wild-type toxin), but the affinity of the toxin was ~100-fold reduced for other members of the K_{Ca}v1 family and also for K_{Ca}v3.1.

In addition to peptide blockers of these channels, several distantly related compounds are reported to block K_{Ca}v1.3 and K_{Ca}v3.1 channels within a range of T-cell activation (17). The binding site and binding stoichiometry of these compounds is very diverse (20, 21), and the blocking mechanism is more complicated than that of the peptide inhibitors. Some blockers bind in the extracellular vestibule of the channel, or at a site located outside the pore region (20), and many of them bind in the inner cavity of the channels below the selectivity filter (21). The best small-molecule inhibitors block K_{Ca}v1.3 channels were synthesized based on structural information of blocker and receptor [reviewed in (17)]. Although correolide derivatives (II) and cyclohexyl-substituted benzamides (21) were found to block T-cell activation and DTH response in vivo, their therapeutic potential is hampered by their modest selectivity for K_{Ca}v1.3 over other members of the K_{Ca}v family.

Recently, a new class of small-molecule K_{Ca}v1.3 inhibitors derived from using 5-methoxypsoralen (5-MOP) (13). 5-MOP is the major K+ channel-blocking material in the extracts of Ruta graveolens; this compound is used clinically in the therapy of psoriasis and is reported to improve the functional deficits in MS patients. Earlier attempts, focused on the reduction of phototoxicity of 5-MOP, resulted in the synthesis H37, of a compound which was effective in reducing the proliferation of myelin-reactive encephalogenic rat T cells but had low affinity for K_{Ca}v1.3 channels (with an EC_{50} of 6.7 pM) (22).

Based on earlier structure-activity relationship studies, Chandy and colleagues replaced the methyl group at the 5-position of 5-MOP with a series phenylalkyl or cyclohexylalkyl sub-
constituents resulting in 12 different compounds (I3). Out of these, 5-(4-phenylbutoxy) psoralen (Psora-4) showed the strongest affinity for Kv1.3 channels (EC_{SO} = 2.9 nM) (Table 1). A detailed analysis of the dose-response curve displayed a Hill coefficient of 2, indicating that more than one molecule interacts with a single Kv1.3 channel, resembling the blocking stoichiometry of PAC (a cyclohexyl-substituted benzamide) (21). Similar to other small-molecule inhibitors of Kv1.3, Psora-4 also binds preferentially to the C-inactivated state of the channel (I1), which is a non-conducting state of the channel entered upon prolonged depolarization. However, in contrast to other small-molecule inhibitors, Psora-4 shows a remarkable selectivity for Kv1.3 over other ion channels of the Shaker family (Kv1.x); it blocks Kv1.1, Kv1.2, Kv1.4, and Kv1.7 channels with 16- to 70-fold lower affinity. In addition, Psora-4 was ineffective against the cardiac Kv1.1 (also termed human ether-a-go-go-related, HERG) channel, the Kv3.1 channel found in murine cytotoxic T cells, and three types of Ca^{2+}-activated potassium channels (i.e., Kv3.3, 1, SKCa3, and BKCa), and had low affinity for the Na_{i}2 sodium channel, which is expressed in mammalian unmethylated and premethylated axons. This remarkable selectivity profile correlates with excellent results in in vitro lymphocyte proliferation assays. The CD3-specific antibody–stimulated proliferation of human T_{EM} cells and myelin basic protein–stimulated proliferation of rat memory T cells are inhibited by Psora-4 with an EC_{SO} of 25 and 60 nM, respectively, as these cells have a Kv1.3^{high} phenotype. On the other hand, Psora-4 was 10-fold less effective in the suppression the proliferation of naive and T_{CM} cells, as these cells escape Kv1.3 inhibition by upregulating Kv3.1. Thus, this new class of Kv1.3 inhibitors fulfills the demand for selective inhibition of the proliferation of a set lymphocytes; it has the highest potency for Kv1.3 inhibition among the small-molecule inhibitors; and it displays a outstanding selectivity profile. The only disadvantage of Psora-4 is its 7.7 nM affinity for Kv1.5—a channel responsible for the ultrarapid delayed–rectifier current in the human heart. This fact initiated in vivo toxicity tests, which show that at 33 mg/kg/day (for five days), subcutaneous injection of Psora-4 is well tolerated in rats. More extensive toxicity studies are required to verify that Psora-4 is safe for in vivo use as an immunosuppressor. Although the selective inhibition of T_{EM} cell proliferation, in principle, could be therapeutically effective in the treatment of MS and other immune diseases related to T_{EM} cells, this possibility has yet to be verified in extensive in vivo animal experiments modeling human diseases.

The central role of Kv1.3 channels in the regulation of T cell activation has been the subject of extensive research in the past. The recent discovery of the Kv1.3^{high} phenotype of encephalogenic T_{EM} cells and the generation of high-affinity and high-specificity blockers of Kv1.3, such as Psora-4, open for therapeutic immunosuppression in autoimmune diseases, without impairing the overall cellular immunity. Other recent publications describing the lateral organization of Kv1.3 channels in the plasma membrane of T cells (23) and the recruitment of Kv1.3 channels into the immunological synapse (24), where antigen presenting cells interact with T cells, also point towards the modulation of immune reactions by Kv1.3 channels. These recent pharmacological and cell biological findings will stimulate further research in this field, which may lead to a Kv1.3-based therapy of immune diseases.

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