# KCa3.1 - a microglial target ready for drug repurposing?

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# $K_{Ca}3.1$ - a microglial target ready for drug repurposing?

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Main Points:

- Drug repurposing for glial cells might accelerate path to patients
- $K_{Ca}3.1$ might be microglia target with an opportunity to repurpose a safe and efficacious drug
- The NIH NCATS Institute provides opportunities to partner academia with industry for drug repurposing

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# Abstract

Over the past decade glial cells have attracted attention for harboring unexploited targets for drug discovery. Several glial targets have attracted *de novo* drug discovery programs, as highlighted in this GLIA Special Issue. Drug repurposing, which has the objective of utilizing existing drugs as well as abandoned, failed, or not yet pursued clinical development candidates for new indications, might provide a faster opportunity to bring drugs for glial targets to patients with unmet needs. Here we review the potential of the intermediate-conductance calcium-activated potassium channels $K_{Ca}3.1$ as the target for such a repurposing effort. We discuss the data on $K_{Ca}3.1$ expression on microglia *in vitro* and *in vivo*. We review the relevant literature on the two $K_{Ca}3.1$ inhibitors TRAM-34 and Senicapoc. Finally, we provide an outlook of what it might take to harness the potential of $K_{Ca}3.1$ as a *bona fide* microglial drug target.

# DRUG REPUROSING FOR GLIAL CELLS

Drug repurposing, also called drug repositioning, has the objective of utilizing existing drugs and failed, abandoned, or not yet pursued clinical development candidates for new indications (Langedijk et al., 2015). This concept has gained increasing traction in the recent years and has been considered for infectious disease, oncology, orphan diseases, as well as CNS indications such as Alzheimer's disease, stroke and pain (Corbett et al., 2012; Corbett et al., 2015; Fagan, 2010; Gupta et al., 2013; Sardana et al., 2011; Sisignano et al., 2015; Wurth et al., 2016). In fact, the National Center for Advancing Translational Sciences (NCATS) at the US National Institutes of Health (NIH) has set up the "Discovering New Therapeutic Uses for Existing Molecules (New Therapeutic Uses)" initiative, which is a "collaborative program designed to develop partnerships between pharmaceutical companies and the biomedical research community to advance development of therapeutics. This innovative program matches researchers with a selection of pharmaceutical industry assets to test ideas for new therapeutic uses, with the ultimate goal of identifying promising new treatments for patients." While there is increasing activity in developing drugs to target glial cells, highlighted by this GLIA Special Issue, could repurposing of existing drugs or clinical-grade compounds accelerate therapeutic opportunities for targeting glia?

Considering that macroglia share the same neurotransmitter receptors as neurons, this question might seem obvious. Indeed, it cannot be excluded that of the multitude of approved drugs targeting glutamate receptors some of their activity might actually originate from glial glutamate receptors (Hubbard et al., 2013; Nicoletti et al., 2015). However, the more specific question is, whether there are drugs or drug-candidates that could be repurposed to address a specific glial hypothesis? Considering that microglia share a molecular repertoire with other immune cells, specifically macrophages and

monocytes, are there any current therapeutic entities which could be repurposed? For example, CD33 antibodies where originally developed against acute myeloid leukemia (AML) (Grosso et al., 2015; Jurcic, 2012). However, the recent interest in CD33 as a microglia target involved in Alzheimer's disease (AD) pathology has sparked interest in these antibodies as potential repurposing opportunity for the treatment of AD (see Wes et al. this GLIA Special Issue).

Ion channels have received considerable consideration as promising drug targets on microglia (Biber et al., 2016; Eder, 2010; Okuse, 2007; Skaper, 2011) Bhattacharya and Biber this GLIA Special Issue). Of these the calcium-activated potassium channel $K_{Ca}3.1$ has received increasing attention (Chen et al., 2015a; Feske et al., 2015; Lam and Wulff, 2011; Maezawa et al., 2012; Staal et al., unpublished observation; Wulff and Castle, 2010).

## $K_{Ca}3.1$

The family of calcium-activated potassium channels (KCa channels) has two groups (Wei et al., 2005) Large conductance (BK, 100-220 pS) channels which are gated by a combination of of intracellular calcium concentration and membrane potential; and the intermediate conductance (IK, 20-85 pS) and small conductance (SK, 2-20pS) channels which are more sensitive to and solely gated by internal calcium ions. The KCNN4 gene coding for $K_{Ca}3.1$ was independently cloned by three groups in 1997 (Ishii et al., 1997; Joiner et al., 1997; Logsdon et al., 1997). It is also known as IK1; IKCa1, ; SK4, SKCa4; and Gardos channel). $K_{Ca}3.1$. is widely expressed in non-excitable cells including immune cells such as T-cells, macrophages and reactive microglia (Feske et al., 2012; Feske et al., 2015; Wulff and Castle, 2010)

## $K_{Ca}3.1$ IN MICROGLIA *in vitro*

Voltage-independent $K_{Ca}3.1$-like $Ca^{2+}$ activated $K^+$ currents were reported first in cultured mouse microglial cells in 1997 (Eder et al., 1997), while expression of $K_{Ca}3.1$ on the mRNA level was confirmed later in rat and mouse microglia *in vitro* (Khanna et al., 2001). Voltage-independent $K_{Ca}3.1$-like $Ca^{2+}$ activated $K^+$ currents could be inhibited by charybdotoxin and clotrimazole, but remained unaffected by paxilline or apamin, inhibitors of large conductance (BK-type) and small conductance (SK-type) $Ca^{2+}$ activated $K^+$ channels, respectively (Schilling et al., 2002; Schilling et al., 2004). Furthermore, charybdotoxin and clotrimazole were found to prevent phorbol 12-myristate 13-acetate (PMA)-induced microglial oxidative burst (Khanna et al., 2001) as well as lysophosphatidic acid-induced microglial migration (Schilling et al., 2004). Together, these data suggested that $K_{Ca}3.1$ is functionally active in microglia and is the predominant $Ca^{2+}$ activated $K^+$ channel in microglia *in vitro*.

Following up on these initial reports, Kaushal et al. (Kaushal et al., 2007) compared expression of $K_{Ca}3.1$ between neuronal, astrocyte and microglial cultures. The reported a much higher mRNA expression in microglial cultures than in neuronal or astrocytic cultures. The expression level was not increased by stimulation with lipopolysaccharide (LPS), a frequently used “activator” of microglial cells. In addition, the authors showed positive immunofluorescence staining for $K_{Ca}3.1$ (rabbit polyclonal $K_{Ca}3.1$/SK4 antibody, Alomone Labs) in microglial cultures. However, staining in neuronal or astrocyte cultures or relevant controls were not shown. Furthermore, using electrophysiology readouts the authors described Ca2+ activated $K^+$ current characteristic of $K_{Ca}3.1$ which was inhibited by the $K_{Ca}3.1.1$ inhibitor TRAM-34 (see section below). In neuronal/microglia co-culture experiments TRAM-34 reduced LPS-induced microglial neurotoxicity, presumably via reduction of p38-dependent nitric oxide (NO) production (Kaushal et al., 2007).

Subsequently, a series of reports from the same group expanded on these observations. Using electrophysiology and TRAM-34 as an inhibitor for $K_{Ca}3.1$ the authors reported that microglia stimulation

with UTP triggered $K_{Ca}3.1$ activation (Ferreira and Schlichter, 2013). The $K_{Ca}3.1$ activation was dependent on substantial increases in intracellular calcium achieved by triggering purinergic-receptor mediated release of calcium from intracellular stores, followed by store-operated calcium entry. Thus, the $K_{Ca}3.1$ activation hyperpolarized the cells and increased the driving force for calcium entry via $Ca^{2+}$-release activated calcium (CRAC) channels and subsequent refilling of intracellular calcium stores (Feske et al., 2012). Furthermore, the authors reported expression of $K_{Ca}3.1$ by immunofluorescence in the rat microglia cell line MLS-9 (anti-$K_{Ca}3.1$ (SK4) rabbit polyclonal antiserum, Abcam); however, no controls were shown. In the second study, IL-4 treatment led to an increase of $K_{Ca}3.1$ mRNA in cultured rat microglia after 6 hours (Ferreira et al., 2014). This correlated with a corresponding increase in the $K_{Ca}3.1$ mediated (TRAM-34 sensitive) potassium current at 24 hours and was maintained for 6 days. The upregulation of $K_{Ca}3.1$ was attributed to the activation of signal transduction pathways involving JAK3 (janus kinase 3), Ras/Raf/MEK (mitogen-activated protein kinase)/ERK (extracellular-signal-regulated kinase), and the transcription factor AP-1 (activating protein-1). T It is important to note that this study provides one of the few examples where $K_{Ca}3.1$ expression levels (i.e. mRNA) were correlated with an orthogonal measurement of $K_{Ca}3.1$ expression, in this case, measurement of the $Ca^{2+}$ activated $K^+$ current sensitive to low concentrations of TRAM-34. Most recently the same group reported $K_{Ca}3.1$ regulation via PKG (protein Kinase G)-dependent pathways in primary rat microglia and the MLS-9 microglia cell line (Ferreira et al., 2015). Using perforated-patch recordings to preserve intracellular signaling, experimental elevation of cGMP increased both the $K_{Ca}3.1$ current and intracellular reactive oxygen species (ROS) production. Both current and ROS were prevented by the kinase inhibitor KT5823 at concentrations of 4-fold over the $K_i$ for PKG, but only ¼ of the $K_i$ of PKA (protein Kinase A). In MLS-9 microglia the ROS-depended $K_{Ca}3.1$ current was also elicited by hydrogen peroxide, inhibited by N-(2-mercaptopropionyl)glycine, a ROS scavenger, and prevented by a the CaMKII ($Ca^{2+}$/calmodulin-

dependent protein kinase II) inhibitor myristolated autocamtide-2 related inhibitory peptide for CaMKII (mAIP) (Ferreira et al., 2015).

A study by Maezawa and colleagues demonstrated that TRAM-34 blocks amyloid beta-induced microglia proliferation, p38MAPK phosphorylation, NFκB (nuclear factor κB) activation, and nitric oxide generation in primary microglia cultures (Maezawa et al., 2011). Furthermore, TRAM-34 inhibited neurotoxic effects of amyloid beta oligomers in mixed microglia-neuron cultures and in organotypic hippocampal slices by decreasing microglial activation and partially preventing synaptic loss (Maezawa et al., 2011).

Taken together, there is convincing evidence for the expression of $K_{Ca}3.1$ in cultured rodent microglia. While the details of regulation of $K_{Ca}3.1$ await confirmation with more specific tool compounds, the currently available data suggest a role for $K_{Ca}3.1$ at the very least in microglia ROS production and signaling.

## $K_{Ca}3.1$ EXPRESSION IN THE CNS – CURRENT DATA AND CHALLENGES

$K_{Ca}3.1$ is widely expressed in peripheral tissues including hematopoietic cells (e.g. erythrocytes platelets, lymphocytes, mast cells, monocytes and macrophages), fibroblasts, vascular endothelial cells and epithelial tissues (reviewed in (Wulff and Castle, 2010)). In contrast, studies reporting the cloning of $K_{Ca}3.1$ (hIK1, hSK4) in tissue, failed to detect its transcript in the CNS with Northern plot (Ishii et al., 1997; Joiner et al., 1997). However, since then several laboratories reported *in vivo* $K_{Ca}3.1$ expression on neurons, astrocytes, oligodendrocytes, astrocytoma cells, neuroblasts, endothelial cells and in CNS tissue extracts by electrophysiology, pharmacology, PCR (polymerase chain reaction), western blot and immunostaining (Boettger et al., 2002; Bouhy et al., 2011; Chen et al., 2011; Engbers et al., 2012; King et al., 2015; Mongan et al., 2005; Turner and Sontheimer, 2014; Turner et al., 2015). Doubts remained,

however, as western blots showed unexpected sizes and contained no proper antibody controls. Furthermore, some of the results were reported as "data not shown" and many of the reports contradicted each other. Be this as it may, surprisingly none of the reports supported expression of $K_{Ca}3.1$ on microglia *in vivo*. Several investigators tried to double label $K_{Ca}3.1$ with microglial markers such as Iba-1, CD11b or tomato lectin, but did not detect any overlap (Bouhy et al., 2011; Turner et al., 2015). One report even went to the trouble, after not being able to detect $K_{Ca}3.1$ *in vivo*, to reconfirm $K_{Ca}3.1$ expression in microglia and macrophages *ex vivo* (Bouhy et al., 2011).

Further complicating the issue of whether or not KCa3.1 is expressed in the CNS, are reports on $Kcnn4^{-/-}$ mice which showed no overt behavioral or neurological phenotype (reviewed in (Wulff and Castle, 2010). These observations, however, were based on gross examination and did not include detailed behavioral tests or closer neurological or neurochemical examinations. In contrast, studies on immune cells from $Kcnn4^{-/-}$ mice displayed a loss of $K_{Ca}3.1$- mediated $K^+$ currents and effects on cellular physiology (e.g. cytokine production) (Di et al., 2010; Lam and Wulff, 2011; Shumilina et al., 2008). However, more recent studies of $KCNN4^{-/-}$ mice reported subtle differences in CNS monoamine levels, increased locomotor activity, alterations in the Y-maze test of working memory as well as differences in ACTH (adrenocorticotropic hormone) release in response to stress (Lambertsen et al., 2012; Liang et al., 2011). Nevertheless, general brain morphology and organization of major brain regions appeared similar and immunofluorescence staining for astrocytes (GFAP) and microglia (CD11b, Iba-1) did not reveal any differences in the morphology or numbers of glial cells between genotypes (Lambertsen et al., 2012). The $KCNN4^{-/-}$ mice were also instrumental in validating some of the $K_{Ca}3.1$ antibodies in peripheral tissues with known high expression levels. (SC-32949, Santa Cruz; AV35098, Sigma; ALM-051, Alamone Labs) (Chen et al., 2015b; Lambertsen et al., 2012). Based on results obtained with these antibodies, the

authors concluded that KCa3.1 is not expressed at immunohistochemically detectable levels in the unperturbed CNS in mice and in post-mortem human brain sections of cortex, cerebellum and brain stem (Lambertsen et al., 2012). Functional voltage-independent $Ca^{2+}$ activated $K^+$ currents were also not detected in patch clamp recordings from microglia in brain slices of healthy juvenile, adult and aged mice (Schilling and Eder, 2007a, 2015).

In contrast to the uninjured CNS, there is evidence that microglia express $K_{Ca}3.1$ under pathological conditions such as ischemic injury (Chen et al., 2011). In non-infarcted areas robust $K_{Ca}3.1$ staining was only observed on brain microvessels. In infarcted tissue, additional $K_{Ca}3.1$ immunoreactivity was detected on small, round, ruffled $CD68^+$ positive cells, which were presumed to be microglia or CNS-infiltrating macrophages. Furthermore, multiple publications have reported beneficial effects of pharmacological inhibitors of $K_{Ca}3.1$ in experimental models of CNS diseases, such as experimental autoimmune encephalomyelitis (EAE), ischemic injury, traumatic brain injury, spinal cord injury, optic nerve transection and microglial CD68 reactivity associated with glioblastoma multiforme (Bouhy et al., 2011; Chen et al., 2011; Chen et al., 2015b; D'Alessandro et al., 2013; Kaushal et al., 2007; Mauler et al., 2004; Reich et al., 2005; Urbahns et al., 2005; Urbahns et al., 2003).

Of high interest is a very recent report in which mice were subjected to middle cerebral artery occlusion with reperfusion (MCAO/R) after 8 days (Chen et al., 2015a). Microglia/macrophages were acutely isolated within 90 min with magnetic CD11b beads and subsequently investigated by electrophysiology. Microglia/macrophages from the infarcted area exhibited higher KCa3.1 current densities than microglia from non-infarcted control brains. A similar increase $K_{Ca}3.1$ current was seen in the brains after intraventricular LPS stimulation. Interestingly, human post mortem tissue from infarcted areas showed KCa3.1 immunoreactivity on hypertrophic, $MAC387^+$ microglia/macrophages. Genetic ablation ($Kcnn4^{-/-}$) or pharmacological blockade of KCa3.1 with TRAM-34 lead to significantly smaller infarct areas on day-8

after MCAO/R, improved neurological deficits, reduced microglia/macrophage activation assessed by Iba-1 staining and reduced brain cytokine levels (e.g. IL-1β (interleukin-1 beta) IFN-γ (interferon gamma), TGF-β1 (Transforming growth factor beta 1)) (Chen et al., 2015a). Finally, in a very elegant experiment the authors showed that TRAM-34 treatment in $Kcnn4^{-/-}$ mice undergoing the same MCAO/R paradigm had no effect. This indicates that TRAM-34 effects were specific to $K_{Ca}3.1$ and suggests that $K_{Ca}3.1$ is a promising target for ischemic stroke (Chen et al., 2015a).

Taken together, there seems to be a consensus that microglia in the unperturbed CNS do not express $K_{Ca}3.1$. However, Under pathological conditions, such as ischemic injury, $K_{Ca}3.1$ is found on rodent and most notably human CNS myeloid cells which might be microglia or infiltrating macrophages. Regardless of their nature of the $K_{Ca}3.1^{+}$ cells, one can make an argument for $K_{Ca}3.1$ potential as an neuroinflammation target (Biber et al., 2016). Considering that microglia *in vitro* resemble a reactive phenotype and that *intra ventricular* LPS application induces $K_{Ca}3.1$ expression *in vivo* (Chen et al., 2015a), one could speculate that in diseases with reactive microglia (Garden and Moller, 2006; Hanisch and Kettenmann, 2007; Kettenmann et al., 2011) $K_{Ca}3.1$ might be expressed and could be exploited as a therapeutic target.

## KCa3.1 INHIBITORS

A number of compounds are now available to study the function of $K_{Ca}3.1$ channels, including toxins and pharmacological $K_{Ca}3.1$ blockers and activators reviewed in detail in (Wulff and Castle, 2010; Wulff et al., 2007). Here we limit our discussion to the two most studied $K_{Ca}3.1$ inhibitors, TRAM-34 and Senicapoc (ICA-17043) with drug-like properties (Figure 1). They were synthesized based on the structure of the antifungal drug clotrimazole (Wulff et al., 2007), a potent blocker of $K_{Ca}3.1$ channels (Alvarez et al., 1992;

Brugnara et al., 1995; Wulff et al., 2001), that was found to be unsuitable for long-term *in vivo* use due to its inhibition of cytochrome P450-dependent enzymes and liver damage (Suzuki et al., 2000; Wulff and Castle, 2010; Zhang et al., 2002).

## TRAM-34

TRAM-34 potently inhibits human $K_{Ca}3.1$ channels with an $IC_{50}$ of 20 nM in recombinant cell lines and has no effect on cytochrome P450-dependent enzymes (Wulff et al., 2000). It has been used to investigate the physiology of $K_{Ca}3.1$ channels in immune cells and the involvement of $K_{Ca}3.1$ channels in several CNS disorders, including multiple sclerosis (Reich et al., 2005), optic nerve transaction (Kaushal et al., 2007), spinal cord injury (Bouhy et al., 2011), ischemic stroke (Chen et al., 2011; Chen et al., 2015b), and glioblastoma multiforme (D'Alessandro et al., 2013).

Kaushal and colleagues showed that cultured microglia produce ROS triggering neuronal death *in vitro* (Kaushal et al., 2007). Using optic nerve transection as an *in vivo* model of neurodegeneration, *intra ocular* injection of TRAM-34 at day 1 and 4 after surgery attenuated neurodegeneration. Given the route of administration, total and free drug concentrations in the vitreous humor or optic nerve were not determined in this study so it is not possible to know whether sufficient free concentrations of TRAM-34 were achieved to block $K_{Ca}3.1$ channels. That said, TRAM-34 not only significantly reduced the neuronal death following optic nerve transection, but also robustly attenuated the microglial activation as assessed by major histocompatibility complex II (MHCII) expression levels. While there are no data proving that it was indeed microglial ROS that damaged the neurons *in vivo*, the data are consistent with the conclusion that inhibition of $K_{Ca}3.1$ attenuated neurodegeneration in mice following transection of the optic nerve.

TRAM-34 has been shown to improve recovery in a model of spinal cord injury (Bouhy et al., 2011). TRAM-34 dose dependently improved locomotor function and reduced tissue loss while increasing neuron and axon sparing although only the highest dose (i.e. 120 mg/kg/day) was consistently significant.

Chen and colleagues evaluated TRAM-34 in a rat model of ischemic stroke (Chen et al., 2011). After administration of TRAM-34 at 10 mg/kg, concentrations in plasma and brain peaked at approximately 2.5 µmol/L between 30 minutes and 1 hour after which it fell to 59 nmol/L in plasma and 191 nmol/L in brain by 12 hours. After administration of TRAM-34 at 40 mg/kg i.p. (intraperitoneal) plasma and brain reached ~1 µmol/L at 8 hours, dropping to 0.4 µmol/L by 12 hours. When given s.c. (subcutaneous), the bio-availability of TRAM-34 was so poor that 120 mg/kg had to be administered in order to get 2.5 µmol/L in plasma. Free plasma concentrations were determined to be approximately 2%. From these data, it is estimated that after 40 mg/kg i.p. at 8 hours the plasma and brain concentrations are 20 nM and 8 nM at 12 hours (before the second dose). At 10 mg/kg i.p. the free plasma and brain concentrations are estimated to be approximately 1 and 4 nM respectively. Thus the TRAM-34 concentrations are at or near the $IC_{50}$ values for $K_{Ca}3.1$ inhibition. Administration of TRAM-34 at both 10 and 40 mg/kg i.p. significantly attenuated the infarct size and the degree of neuronal loss, improved the neurological deficit score and significantly reduced the extent of microglial ED1 staining. Especially promising was the finding that TRAM-34 improved the outcome in this model of stroke even when given 12 hours after the ischemic insult. Current treatments need to be given within 3-4.5 hours (Adams et al., 2007; Del Zoppo et al., 2009), a challenge even when the emergency room is only a short distance away.

Glioblastoma multiforme (GBM) is a diffuse, highly malignant brain tumor known to express $K_{Ca}3.1$, which has been suspected to play a role in the infiltration of the brain paranchyma by these tumor cells, (Weaver et al., 2006). In SCID (severe combined immunodeficiency) mice that had human GL-15

glioblastoma cells xenografted into their brains, TRAM-34 (120 mg/kg i.p.) significantly reduced tumor invasion into the host tissue (D'Alessandro et al., 2013). TRAM-34 levels in brain were determined to be approximately 1400 nM at 2 hours and 400 nM at 12 hours. Assuming that the unbound drug fraction was similar to plasma, free TRAM-34 concentrations would have been approximately 28 to 8 nM, close to the reported $IC_{50}$ values for $K_{Ca}3.1$ inhibition. Furthermore, TRAM-34 reduced the activation of microglia (CD68 positive area) and astrocytes (GFAP positive area). *In vitro* TRAM-34 reduced both phagocytosis and chemotactic activity of primary microglia exposed to GBM-conditioned medium. Together, the data suggest that $K_{Ca}3.1$ inhibitors could be a safe and effective therapy for an otherwise difficult to treat brain tumor.

TRAM-34 (10 and 40 mg/kg) was also evaluated in animal models of epilepsy (Ongerth et al., 2014). It had no effect on behavioral endpoints of the models, but significantly increased hippocampal neurodegeneration and exacerbated neuronal loss. Whether this effect is indeed related to $K_{Ca}3.1$ inhibition or to off-target effects of TRAM-34 is currently unknown.

In summary, in most studies, TRAM-34 was shown to reduce neuroinflammation and provide neuroprotection. While the effects of TRAM-34 reported in the above studies are consistent with inhibition of $K_{Ca}3.1$, off-target effects can not be ruled out. While selective for $K_{Ca}3.1$ over other calcium-activated potassium channels (Wulff et al., 2000), TRAM-34 may inhibit additional targets in microglia which hinders the interpretation of these results (Schilling and Eder, 2007b). Schilling and Eder (2007b) have demonstrated that TRAM-34 blocks lysophosphatidylcholine (LPC)-induced non-selective cation current in primary microglia with a $IC_{50}$ that was similar to its $IC_{50}$ for $K_{Ca}3.1$ channels, while another presumed $K_{Ca}3.1$ blocker charybdotoxin had no effect on LPC signals (Schilling and Eder, 2007b). The identity of TRAM-34 sensitive channels was not determined in this study. Potential candidates could be TRPC6 (transient receptor potential cation 6) and TRPV1 (transient receptor potential vanilloid 1), both

of which are activated by LPC (Schilling and Eder, 2009). In addition, based on the similarity in structures between TRAM-34 and clotrimazole, it is possible that TRPM2 (transient receptor potential melastatin 2) channels, which are blocked by clotrimazole are also inhibited by TRAM-34 (Hill et al., 2004). Hence, TRAM-34 may modulate immune cell function by a mechanism that is unrelated to its inhibition of $K_{Ca}3.1$ channels and further research is needed to identify additional targets of TRAM-34. Moreover, it has recently been demonstrated that TRAM-34 still inhibits some cytochrome P450 isoforms, namely human CYP2B6, CYP2C19 and CYP3A4 with IC values in the low micromolar concentration range (Agarwal et al., 2013). In addition, TRAM-34 shows metabolic instability and has a short half-life (~2 hours in rats and primates) (Maezawa et al., 2012) complicating chronic dosing. Thus, although TRAM-34 is a valuable tool compound, it has issues that may confound interpretation of mechanism in pre-clinical models and may limit its clinical utility. Because of these limitations, replication with other selective $K_{Ca}3.1$ inhibitors (see below) or use of genetically modified animals as done by (Chen et al., 2015a) would be very valuable.

## SENICAPOC (ICA-17403)

Senicapoc (ICA-17043) was initially developed for the treatment of sickle cell anemia and inhibits $K_{Ca}3.1$ channels in human erythrocytes with the $IC_{50}$ of 11 nM (Ataga et al., 2006; Ataga et al., 2011; Ataga et al., 2008; Ataga and Stocker, 2009). Senicapoc works by blocking potassium efflux from erythrocytes subsequently reducing red blood cell dehydration and hemolysis (reviewed in (Wulff et al., 2007)). The drug was well tolerated in Phase 1 clinical trials in both healthy volunteers and in patients with sickle cell disease and had an average plasma half-life of 12.8 days (Ataga et al., 2006; Ataga et al., 2011). In a double blind placebo controlled Phase 2 study, Senicapoc (at 10 mg/day) reduced hemolysis and significantly increased hematocrit and hemoglobin levels in patients with sickle cell disease (Ataga et al.,

2008). In a subsequent Phase 3 trial, Senicapoc was tested for its effects on vaso-occlusive pain crisis (Ataga et al., 2011). However, despite properly engaging erythrocyte $K_{Ca}3.1$, reducing hemolysis and increasing hemoglobin and hematocrit levels, Senicapoc had no effect on pain outcome measures and the trial was terminated (Ataga et al., 2011). While this in fact constitutes a clinical trial with negative outcome, in retrospect it seems likely that rather than a failed drug, the underlying hypothesis (i.e. reduction sickle cell dehydration and hemolysis may lead to improvements in vaso-occlusive crisis occurring during the sickle cell disease) might have been flawed.

While the peripheral pharmacokinetics of Senicapoc have been described in great detail (McNaughton-Smith et al., 2008), its ability to cross the blood-brain barrier was only recently investigated (Staal et al., unpublished observations). After 10 mg/kg oral dosing in rats, Senicapoc achieved free plasma concentrations of 17 nM and 65 nM and free brain concentrations of 37 and 136 nM at 1 and 4 hours post-dose respectively. Cerebro-spinal fluid (CSF) concentrations were determined to be 25 nM and 121 nM at 1 and 4 hours post-dosing which are in-line with the free brain concentrations. These data suggest that Senicapoc achieves CNS concentrations are greater than its $IC_{50}$ value for $K_{Ca}3.1$ channels and thus should be sufficient to inhibit it (McNaughton-Smith et al., 2008). In the same study, Senicapoc's selectivity was assessed in a screen of ~70 additional related targets (Staal et al., unpublished observations). None of the targets tested was inhibited by Senicapoc at 10 μM, providing additional evidence that Senicapoc is selective for $K_{Ca}3.1$ channels (Staal et al., submitted). Sinacepoc was tested in the chronic constriction injury model of neuropathic pain (Bennett and Xie, 1988). Senicapoc dose dependently (10, 30 and 100 mg/kg p.o. (per os, oral administration)) attenuated the mechanical hypersensitivity induced by the peripheral nerve injury, although only the highest dose was significant. Furthermore, in contrast to reported locomotor effects in $Kcnn4^{-/-}$ mice (Lambertsen et al., 2012), the authors did not observe any significant impact of Senicapoc on locomotor activity. This is significant as standard of care treatments for neuropathic pain, such as gabapentin and pregabalin, cause significant

sedation (Brix Finnerup et al., 2013). While the study does not shed light on the cell types in the CNS that express $K_{Ca}3.1$, it clearly demonstrates that Senicapoc was efficacious in ameliorating pain behaviors in rats with peripheral nerve injury and these conclusions were supported by the free drug concentrations attained in plasma, brain and CSF.

## NEXT STEPS FOR $K_{Ca}3.1$ AS A MICROGLIA TARGET

The availability of Senicapoc as a clinically ready, CNS penetrant $K_{Ca}3.1$ inhibitor should be a considerable boost to investigations in the field. Even more so, as Senicapoc is part of the NCATS "New Therapeutic Uses" initiative (https://ncats.nih.gov/ntu/assets/2012). NCATS will provide access to the industrial partner and the asset (i.e. Senicapoc) as well as the potential for funding. Interestingly, the NCATS list of assets for repurposing has a basic assessment scale of CNS penetrance the available compounds. For Senicapoc the CNS penetrance is listed as "unknown". While this might have limited the interest in repurposing for CNS indications in the past, the unexpected CNS penetrance of this drug (Staal et al., unpublished observations), might provide the necessary impetus for future activities.

What does the availability and CNS penetrance of Senicapoc mean for $K_{Ca}3.1$ as a microglia target? The available information of *bona fide* microglia $K_{Ca}3.1$ expression in CNS disorders is still limited to ischemic injury (Chen et al., 2015a). Based on the robust expression on microglia *in vitro* and the LPS-induced upregulation of $K_{Ca}3.1$ in vivo, one might speculate that other CNS disorders with disease-associated microglial phenotypes could also induce expression of $K_{Ca}3.1$. Such data, for example in AD, Parkinson's and Huntington's Disease, as well as psychiatric conditions such as depression or schizophrenia, are urgently needed. Furthermore, the availability of molecule which binds to $K_{Ca}3.1$ with high affinity and selectivity might provide additional options for detection of $K_{Ca}3.1$. Labeling these (or similar)

compounds could be an alternative to antibodies in species for which no validated antibodies are available.

Taken together the currently available evidence suggests that $K_{Ca}3.1$ might be an interesting drug repurposing opportunity for a microglia target. In contrast to targets discussed in this GLIA Special Issue, a clinically safe and efficacious $K_{Ca}3.1$ blocker is readily available. $K_{Ca}3.1$ is in the curious position of having the validated biology lagging behind the availability of a clinically ready drug. In our mind this awards an outstanding opportunity to use Senicapoc as a chemical probe to validate microglia $K_{Ca}3.1$ (Bunnage et al., 2013; Frye, 2010). While Senicapoc's patent life is too short to be exploited commercially, Senicapoc is an outstanding compound to provide proof of concept data in humans. Further investigations of $K_{Ca}3.1$ as a target, either by academic, non-for-profit or for-profit entities will depend on the quality of proof of concept data. If experiments are carefully planned, diligently executed and judiciously interpreted (see Möller & Boddeke this GLIA Special Issue) $K_{Ca}3.1$ inhibitors might make targeting glial cells for CNS disorders a reality, sooner rather than later.

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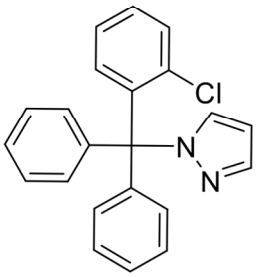
# FIGURES

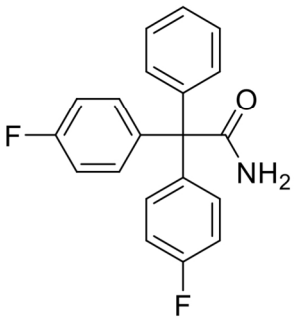
Figure 1: Structures of the two $K_{Ca}3.1$ inhibitors TRAM-34 and Senicapoc with their $IC_{50}$s for human $K_{Ca}3.1$ channels.

# CONFLICT OF INTEREST

Elena Dale, Roland G.W. Staal and Thomas Möller are former employees of Lundbeck Research USA. They are not connected to any current drug discovery programs on KCa3.1 or any of its inhibitors.




**TRAM-34**
$IC_{50}$ ≈ 20 nM




**Senicapoc (ICA-17043)**
$IC_{50}$ ≈ 11 nM

**Figure 1.** Structures of the two $K_{Ca}3.1$ inhibitors TRAM-34 and Senicapoc with their $IC_{50}$s for human $K_{Ca}3.1$ channels.

595x793mm (72 x 72 DPI)