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Review

Cinnabarinic acid and xanthurenic acid: two unorthodox kynurenine metabolites that interact with metabotropic glutamate receptors.

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Abstract

Cinnabarinic and xanthurenic acids are kynurenine metabolites generated by oxidative dimerization of 3-hydroxyanthranilic acid and transamination of 3-hydroxykynurenine, respectively. Recent evidence suggests that both compounds can affect brain fuction and neurotransmission and interact with metabotropic glutamate (mGlu) receptors. Cinnabarinic acid behaves as an orthosteric agonist of mGlu4 receptors, whereas some of the effects produced by xanthurenic acid in the CNS involve mGlu2 and mGlu3 receptors. Cinnabarinic acid could play an important role in mechanisms of neuroinflammation acting as a linking bridge between the immune system and the CNS. Xanthurenic acid has potential implications in the pathophysiology of schizophrenia and is a promising candidate as a peripheral biomarker of the disorder. The action of cinnabarinic acid and xanthurenic acid may extend beyond the regulation of mGlu receptors and may involve several diverse molecular targets, such as the aryl hydrocarbon receptor for cinnabarinic acid and vesicular glutamate transporters for xanthurenic acid. The growing interest on these two “unorthodox” metabolites of the kynurenine pathway may unravel new aspects in the complex interaction between tryptophan metabolism and brain function, and lead to the discovery of new potential targets for the treatment of neurological and psychiatric disorders.

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Key words: cinnabarinic acid **-** xanthurenic acid **-** metabotropic glutamate receptors – neuroinflammation - schizophrenia

1. Introduction

The kynurenine pathway of tryptophan metabolism generates a series of neuroactive compounds of which quinolinic acid and kynurenic acid gained popularity in neuroscience for their ability to activate and inhibit NMDA receptors, respectively (Stone and Perkins, 1981; de Carvalho et al. 1996; Parsons et al., 1997; Schwarcz et al., 2012). The interest in these two metabolites gave the wrong impression amongst neuroscientsts that the kynurenine pathway was an « inverse L », with the central metabolite, L-kynurenine, giving raise to kynurenic acid, or, alternatively, to a sequential series of metabolites that include 3-hydroxykynurenine, 3-hydroxyanthranilic acid, and quinolinic acid. A great emphasis was placed on two key enzymes, kynurenine monooxygenase (KMO) and kynurenine amino transferase, which drive the metabolic fate of L-kynurenine and tip the balance between kynurenic acid and quinolinic acid (reviewed by Schwarcz et al., 2012). Compounds that are generated « horizontally » by 3-hydroxykynurenine and 3-hydroxyanthranilic acid, i.e. xanthurenic acid and cinnabarinic acid, respectively (Fig. 1), have been considered as « by products » of the kynurenine pathway, with little or no interest for the physiology and pathology of the CNS. However, recent findings suggest that cinnabarinic acid and xanthurenic acid are neuroactive compounds, and that their actions modulate, directly or indirectly, metabotropic glutamate (mGlu) receptors. These receptors form a family of eight subtypes, of which the mGlu4 receptor is targeted by cinnabarinic acid, whereas mGlu2 and mGlu3 receptors are involved in the action of xanthurenic acid (see below). mGlu2, mGlu3, and mGlu4 receptors are coupled to Gi/Go proteins and are preferentially localized at presynaptic nerve terminals, where they negatively regulate neurotransmitter release (reviewed by Nicoletti et al., 2011). mGlu4 receptors are also expressed by antigen-presenting cells, and their activation drives T cell differentiation into regulatory T (Treg) cells, thereby restraining autoimmunity and neuroinflammation (Falarino et al., 2010). The interaction with mGlu receptors, as well as other emerging mechanisms (e.g. inhibition of vesicular glutamate transporters by xanthurenic acid and activation of the aryl hydrocarbon - Ah

- receptor by cinnabarinic acid) have generated new interest in these two « unorthodox » kynurenine metabolites. Cinnabarinic acid (2-amino-3-oxo-3H-phenoxazine-1,9-dicarboxylic acid), which is responsible for the anitimicrobial activity of the fungus, *Pycnoporus cinnabarinus* (Eggert et al., 1997), is generated from enzymatic and non-enzymatic oxidation of 3-hydroxyanthranilic acid (Rao, 1966; Ogawa et al., 1983; Christen et al., 1992). Xanthurenic acid is formed by transamination of 3-hydroxykynurenine (Malina and Martin, 1996). In rat and human brain, transamination of 3-hydroxykynurenine into xanthurenic acid is catalyzed by type-2 kynurenine aminotransferase (Sathysaikumar et al., 2014), the same enzyme that converts kynurenine into kynurenic acid (reviewed by Schwarcz et al., 2012).

This review will focus on recent findings highlighting a potential role for cinnabarinic acid and xanthurenic acid in CNS physiology and pathology focusing on the possible involvement of mGlu receptors in the mechanism of action of these compounds.

2. Cinnabarinic acid

Recent findings led to the identification of two novel receptor targets for cinnabarinic acid: (i) the mGlu4 receptor; and (ii) the aryl hydrocarbon (Ah) receptor. Interestingy, both receptors have been implicated in mechanisms that lie at the core of neuroinflammation, by regulating the bidirectional communication between antigen presenting cells (APCs) and T lymphocytes at the immunological synapse (reviewed by Volpi et al., 2012). In collaboration with the research groups of Jean-Phlippe Pin and Cyrille Goudet (University of Montpellier, France), and Francine Acher (University Renè Descartes, Paris, France) we have found that cinnabarinic acid behaves as a weak orthosteric agonist of mGlu4 receptors, with no activity at other mGlu receptor subtypes (Fazio et al., 2012). mGlu4 receptors are coupled to Gi/o GTP-binding proteins, and are localized on presynaptic terminals where they negatively regulate neurotransmitter release (reviewed by Nicoletti et al., 2011). Selective positive allosteric modulators (PAMs) of mGlu4 receptors are under development for the treatment of Parkinson’s disease (reviewed by Nickols and Conn, 2014; Walker and Conn,

2015), and are potential candidate drugs for the treatment of neuropathic pain (Goudet et al., 2008). Of note, mGlu4 receptors are also expressed by APCs and T lymphocytes (Fallarino et al., 2010; see below).

Cinnabarinic acid acts as a partial agonist in cell clones expressing mGlu4 receptors by interacting with the glutamate binding pocket localized in the N-terminus Venus Fly Trap domain of the receptor. In addition, cinnabarinic acid inhibits cAMP formation in cultured cerebellar granule cells, which are known to express large amounts of mGlu4 receptors (Santi et al., 1994), and the cAMP response to low concentrations of cinnabarinic acid is abolished in cultures prepared from mGu4 receptor knockout mice (Fazio et al., 2012). Cinnabarinic acid attenuates excitotoxic neuronal death in cultured cortical cells, and protects nigro-striatal neurons against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) toxicity when locally infused into the mouse external globus pallidus (Fazio et al., 2012), thus mimicking the action of conventional mGlu4 receptor agonists (Maj et al., 2003; Battaglia et al., 2006). These findings indicate that cinnabarinic acid is able to activate native mGlu4 receptors in the CNS. However, most of the effects seen in cultured neurons require concentrations of cinnabarinic acid >10-30 μM (Fazio et al., 2012), and this casts doubt on the physiological relevance of the interaction beween cinnabarinic acid and neuronal mGlu4 receptors. Under normal conditions, brain cinnabarinic acid is barely detectable in the mouse brain, but its levels dramatically increase (up to >150 pg/mg tissue) in response to systemic injection of lipopolysaccharide (LPS), which is known to cause neuroinflammation (Fazio et al., 2012). It is possible that cinnabarinic acid formed as a by-product of the kynurenine pathway activated in response to neuroinflammation serves as an endogenous protective agent that restrains synaptopathy and neuronal damage caused by pro-inflamatory cytokines secreted by immune cells or by resident astrocytes and microglia. This attractive hypothesis warrants further investigation.

The evidence that the mGlu4 receptor is involved in mechanisms regulating the fate of T cells at the immune synapse (Fallarino et al., 2010) has provided new insights into the role played by mGlu4 receptors in neuroinflammation. The mGlu4 receptor is expressed in dendritic cells, and its

activation tips the balance of Th cell differentiation in favour of a Treg immune tolerant phenotype. As a result of this mechanism, systemic treatment with the mGlu4 receptor PAM, (-)-N-phenyl-7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxamide (PHCCC), protects mice against the development of experimental autoimmune encephalomyelitis (EAE, an experimental animal model of multiple sclerosis), whereas mice lacking mGlu4 receptors show a more severe form of EAE in response to immunization with a myelin-related antigen (Fallarino et al., 2010). A more recent study has confirmed these findings showing that compound ADX88178, a novel mGlu4 receptor PAM endowed with high potency and selectivity and optimized PK profile, protects mice against EAE and stimulates dendritic cells to produce tolerogenic cytokines, such as interleukin-10 and transforming growth factor-β *via* a Gi-independent signaling pathway that involves phosphatidylinositol-3-kinase, the tyrosine kinase, Src, and type-1 indoleamine 2,3-dioxygenase (IDO-1) (Volpi et al., 2016). The evidence that activation of mGlu4 receptors directs Th differentiation toward Treg cells at the expense of Th17 cells (Falarino et al., 2010) laid the groundwork for the study of cinnabarinic acid in EAE mice. Systemic treatment of cinnabarinic acid (0.1-10 mg/kg, i.p.) protected against MOG_{35-55} (fragment 35-55 of the mlyelin oligodendrocyte glycoprotein)-induced EAE by boosting an immune response dominated by Treg cells (Fazio et al., 2014). However, this action of cinnabarinic acid was only partially reduced in mGlu4 knockout mice, suggesting that cinnabarinic acid recruits additional targets to regulate immune function and restrain neuroinflammation. A growing body of evidence indicates that the Ah receptor is a major target for cinnabarinic acid in the modulation of the immune system. The Ah receptor is a ligand-dependent transcription factor that owes its name to its interaction with aromatic hydrocarbons, such as benzopyrene, 3-methylcolanthrene, and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Binding of aromatic hydrocarbons to Ah receptors induces the expression of genes encoding for enzymaes involved in xenobiotic metabolism, such as CYP1A1 and CYP1A2. Besides this established function, the Ah receptor has an emerging role in the regulation of the immune system promoting T cell differentiation into Treg or Th17 cells depending on the receptor ligand (reviewed

by Fallarino et al., 2014). Recent evidence indicates that Ah receptor activation mediates immune tolerance to bacterial endotoxins *via* IDO-1 signaling activity (Bessede et al., 2014). The Ah receptor is targeted by a series of tryptophan metabolites that includes L-kynurenine and kynurenic acid (Mezrich et al., 2010; Nguyen et al., 2010; Maaetoft-Udsen et al., 2012; Stephens et al., 2013; Kawasaki et al., 2014; Nuti et al., 2014). Using the expansion of interleukin-22 producing cells as a read-out system, Lowe et al. (2014) have found that cinnabarinic acid acts as an endogenous agonist of Ah receptors. Interestingly, cinnabarinic acid was more effective than L-kynurenine or kynurenic acid in inducing interleukin-22 but less effective in inducing CYP1A1 in mouse or human T lymphocytes (Lowe et al., 2014). Thus, by analogy with estrogen and progesterone receptor ligands, cinnabarinic acid might be defined as a selective Ah receptor modulator (SAhRM) (Lowe et al., 2014). Mechanisms of ligand- and signaling-bias at the Ah receptors might drive the pattern of the cellular response of the Ah receptor to different kynurenine metabolites in the regulation of xenobiotic metabolism and immune function. It will be interesting to establish how mGlu4 and Ah receptors contribute to the overall response of immune cells to cinnabarinic acid taking into account that other endogenous ligands, such as glutamate for mGlu4 and tryptophan metabolites for Ah receptors, might compete with cinnabarinic acid for receptor binding, thereby shaping the cellular response to cinnabarinic acid.

Interestingly, activation of Ah receptors by cinnabarinic acid protects cultured hepatocytes against apoptosis induced by ethanol, hydrogen peroxide, and thapsigargin. This effect is mediated by the induction of stanniocalcin-2, a putative secreted glycophosphoprotein that has shown protective activity against endoplasmic reticulum and oxidative stress (Joshi et al., 2015). Whether this mechanism contributes to the neuroprotective activity of cinnabarinic acid against excitotoxic neuronal death (Fazio et al., 2012) remains to be determined.

3. Xanthurenic acid

Xanthurenic acid has been the subject of multidisciplinary studies, which address *inter alia* its role in apoptotic cell death (Malina et al., 2001 ; Malina and Hess, 2004), regulation of natriuresis (Cain et al., 2007 ; Hoffman et al., 2013), regulation of insulin secretion and activity (Oxenkrug et al., 2013), and activation of gametogenesis in *Plasmodium* species (Garcia et al., 1998). Gobaille et al. (2008) have shown that xanthurenic acid meets the criteria to be considered as a putative neurotransmitter. Xanthurenic acid is present in micromolar amounts in the rat brain, is stored in synaptic vesicles, released by depolarizing stimuli in a Ca^{2+}-dependent fashion, and actively transported by a neuronal sodium/chloride symporter (Gobaille et al., 2008). [^{3}H]Xanthurenic acid binds to specific and saturable recognition sites in brain membranes, and specifically bound [^{3}H]xanthurenic acid is displaced by picolinic acid and, to a lesser extent, by kynurenic acid and quinolinic acid (Taleb et al., 2012). In addition, low micromolar concentrations of xanthurenic acid enhance [^{35}S]GTP-γ-S binding in crude synaptosomal preparations, suggesting that xanthurenic acid binds to a G-protein coupled receptor (Taleb et al., 2012). The identity of this receptor is unknown at present. A series of studies have examined the possibility that xanthurenic acid interacts with group-II mGlu receptors (mGlu2 and mGlu3 receptors). Intravenous injection or local iontophoretic application of xanthurenic acid reduced sensory inhibition of ventrobasal thalamic neurons mediated by the activation of reticular thalamic neurons (Copeland et al., 2013). Xanthurenic acid only partially mimicked the action of the prototypical mGlu2/3 receptor agonist, (1S,2S,5R,6S)-2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylic acid (LY354740), because the action of both compounds was attenuated by the preferential mGlu2/3 receptor antagonist, 2-[(1S,2S)-2-carboxycyclopropyl]-3-(9H-xanthen-9-yl)-D-alanine (LY341495), but only the action of LY354740 was potentiated by the mGlu2 receptor enhancer, N-(4-(2-methoxyphenoxy)phenyl)-N-(2,2,2-trifluoroethylsulfonyl)pyrid-3-ylmethylamine (LY487379) (Copeland et al., 2013). In contrast, inhibition of synaptic transmission by xanthurenic acid was insensitive to LY341495 in rat hippocampal slices, and, as opposed to LY354740, xanthurenic acid failed to reduce paired-pulse depression in the hippocampal dentate gyrus (Neale et al., 2013). It was concluded that xanhurenic

acid does not directly interact with mGlu2 or mGlu3 receptors (see below). However, xanthurenic acid displayed a high potency in activating mGlu2 and mGlu3 receptors in transfected HEK293 cells showing no activity at recombinant mGlu4- or mGlu7 receptors (Fazio et al., 2015). In mouse cortical cells, xanthurenic acid mimicked the action of mGlu2/3 receptor agonists in inhibiting cAMP formation, and its action was sensitive to LY341495 and attenuated by genetic deletion of mGlu2 receptors (Fazio et al., 2015). Specifically bound [^{3}H]xanthurenic acid was not displaced by mGlu2/3 receptor ligands in brain membranes, and xanthurenic acid did not inhibit [^{3}H]LY341495 binding in brain membranes (Fazio et al., 2015) and in membranes stably expressing human mGlu2 receptors (Neale et al., 2013). Interestingly, however, specific [^{3}H]xanthurenic acid binding could be detected in membranes prepared from mGlu2- or mGlu3-expressing HEK-293 cells, but not in membranes prepared from mock cells or from mGlu4-expressing cells (Fazio et al., 2015). It is difficult to formulate a single hypothesis that may uniformely explain all these findings. It appears clear that xanthurenic acid does not directly interact with the glutamate binding pocket of mGlu2 and mGlu3 receptors. It cannot be exlluded that xanthurenic acid acts as a allosteric agonist (i.e., an allosteric compound endowed with intrinsic efficacy) of mGlu2/3 receptors, but this does not explain why inhibition of field excitatory post-synaptic potentials (fEPSP) by xanthurenic acid in hippocampal slices was insensitive to pharmacological blockade of mGlu2/3 receptors (Neale et al., 2013). Xanthurenic acid might interact with its own receptor (perhaps a not-yet identified G-protein coupled receptor), which may engage mGlu2/3 receptor signaling and might also signal *via* other mechanisms. Perhaps, the most likely explanation for some of the observed effects of xanthurenic acid (Neale et al., 2013) is that the compound targets other mechanisms regulating glutamatergic neurotransmission. Interestingly, xanthurenic acid inhibits vesicular glutamate transporters (VGLUTs) (Bartlett et.al. 1998), thereby preventing glutamate uptake into synaptic vesicles (see Neale et al., 2014). A series of structurally unrelated VGLUT inhibitors, such as Rose Bengal, Congo Red, and Chicago Sky Blue 6B, shares with xanthurenic acid the ability to depress fEPSPs at the Schaffer collateral-CA1 pyramidal cell synapses (Neale et al., 2014), and to reduce the

amplitude of fEPSPs recorded in the dentate gyrus (Neale et al., 2013). Thus, it is possible that, at least in distinct CNS regions, xanthurenic acid inhibits glutamatergic transmission by blocking transport of L-glutamate into synaptic vescicles thereby ultimately reducing the synaptic release of L-glutamate.

The mGlu2 receptor has been implicated in the pathophysiology of schizophrenia (Moghaddam and Adams, 1998; Aghajanian and Marek, 2000; Marek et al., 2000; Gonzales-Maeso et al., 2008; Moreno et al., 2016; Holloway et al., 2013; reviewed by Delille et al., 2013), and both mGlu2/3 orthosteric agonists and mGlu2 receptor PAMs show efficacy in experimental animal models that are predictive of antipsychotic activity (Fell et al., 2012; Hiyoshi et al., 2014; Walker and Conn, 2015). An exploratory analysis of clinical studies showed that pomeglumetad methionyl (the oral prodrug of the mGlu2/3 receptor agonist, LY404039) was effective in relieving both positive and negative symptoms in subgroups of patients affected by schizophrenia who were early in disease and had not been previously treated with atypical antipsychotics (Kinon et al., 2015). Xanthurenic acid displayed antipsychotic-like activity in mice challenged with [5R,10S]-[+]-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine (MK-801) *via* the activation of mGlu2 receptors (Fazio et al., 2015). Interestingly, bood levels of xanthurenic acid were substantially reduced in a large cohort of patients affected by schizophrenia. This reduction persisted after months of treatment with antipsychotic drugs and was also observed in first-degree relatives of patients affected by schizophrenia (Fazio et al., 2015). Other kynurenine metabolites that lie downstream of KMO, such as 3-hydroxykynurenine, 3-hydroxyanthranilic acd, and quinolinic acid were also reduced in the blood of patients affected of schizophrenia, as expected (Erhardt et al., 2001; Linderholm et al., 2012; Schwarcz et al., 2012; Kegel et al., 2014), but only xanthurenic acid and 3-hydroxyanthranilic acid were reduced in first-degree relatives of patients (Fazio et al., 2015). These findings suggest that (i) a reduction of xanthurenic acid production might contribute to the pathophysiology of schizophrenia by restraining the endogenous activation of mGlu2 receptors; and, (ii) blood levels of xanthurenic acid might represent a new peripheral biomarker that may

allow the indentification of individuals at risk of developing schizophrenia. Such individuals might then be able to receive disease-modifying treatments in the early phases of the disorder. Blood levels of xanthurenic acid are also lower in patients affected by attention deficit and hyperactivity disorder (ADHD) (Aarsland et al., 2015) and cluster headache (Curto et al., 2016a), and are higher in patients affected by chronic migraine (Curto et al., 2016b). However, these changes are small and their biological significance is uncertain.

The discovery that xanthurenic acid is a potent inhibitor of sepiapterin reductase (Haruki et al., 2016) indicates that the function of xanthurenic acid in the CNS extends beyond the regulation of membrane transporters or neurotransmitter receptors. Sepiapterin reductase is the final enzyme in the *de novo* synthesis of tetrahydrobiopterin, the enzymatic cofactor of tyrosine hydroxylase, tryptophane hydroxylase, and nitric oxide synthase. This mechanism might limit the stimulation of tetrahydrobiopterin synthesis caused by pro-inflammatory cytokines (which also induce the kynurenine pathway), and might aso contribute to explain the psychiatric adverse effects of interferon treatment (Haruki et al., 2016). Inhibition of tetrahydrobiopterin synthesis by xanthurenic acid might also pave the way to the design of new drugs aimed at restraining dopamine or serotonin synthesis in psychiatric disorders such as drug addiction and schizophrenia.

Conclusions

It is becoming clear that many members of the kynurenine pathways can have complex effects in physiological systems both within the brain and in other parts of the body. In particular, both cinnabarinic acid and xanthurenic acid appear to be able to affect several diverse molecular targets and signaling systems that are only beginning to be uncovered. What is clear, however, is that these compounds can affect brain function and neurotransmission in a variety of ways, and that this may be altered under pathological conditions. Thus, future studies on the function of cinnabarinic acid and xanthurenic acid will no doubt result in better understanding of disease processes and may reveal new potential therapeutic targets in nervous system diseases.

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Figure legend

Fig. 1 – The kynurenine pathway with highlight on cinnabarinic acid and xanthurenic acid.

IDO = indoleamine 2,3-dioxygenase ; TDO = tryptophan 2,3-dioxygenase ; KATs = kynurenine aminotransferases ; KMO = kynurenine monooxygenase ; 3-HAO = 3-hydroxyanthranilic acid 3,4-dioxygenase.

Figure(s)

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