Tetrahydrobiopterin Biosynthesis as a Potential Target of the Kynurenine Pathway Metabolite Xanthurenic Acid*

Received for publication, July 24, 2015, and in revised form, November 5, 2015
Published, JBC Papers in Press, November 12, 2015, DOI 10.1074/jbc.C115.680488
Hirohito Haruki1, Ruud Hovius, Miriam Gronlund Pedersen2, and Kai Johnsson3
From the Institute of Chemical Sciences and Engineering, Institute of Bioengineering, National Centre of Competence in Research (NCCR) in Chemical Biology, École Polytechnique Fédérale de Lausanne (EPFL), 1015 Lausanne, Switzerland

Tryptophan metabolites in the kynurenine pathway are up-regulated by pro-inflammatory cytokines or glucocorticoids, and are linked to anti-inflammatory and immunosuppressive activities. In addition, they are up-regulated in pathologies such as cancer, autoimmune diseases, and psychiatric disorders. The molecular mechanisms of how kynurenine pathway metabolites cause these effects are incompletely understood. On the other hand, pro-inflammatory cytokines also up-regulate the amounts of tetrahydrobiopterin (BH₄), an enzyme cofactor essential for the synthesis of several neurotransmitter and nitric oxide species. Here we show that xanthurenic acid is a potent inhibitor of sepiapterin reductase (SPR), the final enzyme in de novo BH₄ synthesis. The crystal structure of xanthurenic acid bound to the active site of SPR reveals why among all kynurenine pathway metabolites xanthurenic acid is the most potent SPR inhibitor. Our findings suggest that increased xanthurenic acid levels resulting from up-regulation of the kynurenine pathway could attenuate BH₄ biosynthesis and BH₄-dependent enzymatic reactions, linking two major metabolic pathways known to be highly up-regulated in inflammation.

Tetrahydrobiopterin (BH₄) is a cofactor for the tyrosine, tryptophan, and phenylalanine hydroxylases and alkylglycerol monoxygenase as well as nitric oxide synthases (1). These BH₄-dependent enzymes play essential roles in synthesis of signaling molecules such as dopamine, serotonin, and nitric oxide in animals.

BH₄ levels are constitutively high in cells carrying out aromatic amino acid hydroxylation (for example, dopamine- and serotonin-producing cells in brain or intestine and phenylalanine-hydroxylating cells in liver), but also up-regulated in certain cell types by stimulation with pro-inflammatory cytokines (2). The latter observation suggests that BH₄ plays a role in inflammatory processes. Furthermore, accumulating evidence shows that increased levels of BH₄ intensify pain sensitivity, whereas reduction of BH₄ biosynthesis is an efficient strategy to diminish neuropathic and inflammatory pain (3–5).

BH₄ is synthesized de novo from GTP by the sequential action of three enzymes, of which sepiapterin reductase (SPR) is the last (see Fig. 1A) (1). The last step can also be catalyzed by alternative enzymes such as aldose reductase and carbonyl reductase (6). SPR furthermore reduces sepiapterin to dihydrobiopterin (BH₂), which is subsequently reduced to BH₄ by dihydrofolate reductase in the salvage pathway of BH₄ synthesis.

We have reported that the anti-inflammatory/immunosuppressive drug sulfasalazine and its metabolite sulfapyridine are potent inhibitors of SPR, and we proposed that the mechanism of action of these two sulfonamides in rheumatoid arthritis involves SPR inhibition and interference with BH₄ biosynthesis (7). Recently, we performed a screen for additional SPR inhibitors among clinically approved drugs, which revealed that various sulfa drugs are potent inhibitors of human SPR, both in vitro and in cell culture experiments (8). The decrease of BH₄-dependent neurotransmitter biosynthesis in cell-based assays by sulfa drugs furthermore provides a rationale for some of their CNS-related side effects.

We now report that the kynurenine pathway metabolite xanthurenic acid is a potent SPR inhibitor, providing a link between two major metabolic pathways known to be highly up-regulated in inflammation.

**Experimental Procedures**

Small molecule screening and SPR enzymatic assays were performed as described (8). The natural products and bioactive compounds analyzed for SPR inhibition are part of a commercially available 1040-compound library (NINDS2, MicroSource). N-terminally hexahistidine-tagged SPR from human, mouse, and rat was used for enzymatic assays. The recombinant proteins were prepared as described (7). Human SPR was purified and cocrystallized with xanthurenic acid as described (5), except that SPR was eluted with xanthurenic acid in the presence of NADP⁺ and that 1.9 M ammonium sulfate was used for crystallization. The structure was solved by molecular replacement using 4HWK (8) as a search model and refined as described in Ref. 5. For isothermal titration calorimetry experiments, an N-terminal hexahistidine-tagged human SPR was expressed in BL21(DE3) Escherichia coli, purified to homogeneity by Ni²⁺-nitrilotriacetic acid chromatography (7), and subjected to chromatography on a HiTrap Blue HP resin (GE Healthcare) to obtain SPR devoid of any cofactor, as verified by the absence of absorbance bands at 260 and 340 nm. Isothermal titration calorimetry was performed using an ITC200 (GE
Results and Discussion

To identify natural SPR inhibitors whose role might be a regulation of BH₄ biosynthesis, we screened a collection of selected natural products and bioactive compounds for binding to human SPR. We identified two tryptophan metabolites as SPR inhibitors, xanthurenic acid and N-acetylserotonin, with potential physiological relevance (see below). N-Acetylserotonin is a metabolite in the pathway of synthesis of melatonin (Fig. 1B). In fact, the inhibitory activity of N-acetylserotonin toward SPR (Kᵢ = 200 nM for rat SPR) was discovered by Katoh et al. (9) three decades ago, and it has been used as tool compound for controlling SPR activity (10). Furthermore, N-acetylserotonin has been used as the starting point for the recent development of the potent SPR inhibitor SPRi3 (Fig. 2), which has been shown to reduce neuropathic and inflammatory pain in mice through a reduction of BH₄ levels (5). It has been speculated that the physiological role of inhibition of SPR by N-acetylserotonin is negative feedback regulation, if any, as the initial enzyme in the pathway is BH₄-dependent tryptophan hydroxylase (11, 12).

Xanthurenic acid on the other hand is a metabolite in the major tryptophan-catabolizing kynurenine pathway (Fig. 1B), in which all enzymes are BH₄-independent. Inhibition of BH₄ biosynthesis by any of the metabolites in the kynurenine pathway has not been reported. The physiological role of xanthurenic acid in mammals remains elusive. Xanthurenic acid has been frequently described as the inert end product of the detoxification of 3-hydroxykynurenine (13, 14). Depending on the conditions, xanthurenic acid has been shown to act in vitro as either an anti-oxidant or a pro-oxidant, scavenger or generator of reactive oxygen species, and as a photosensitizer (15). A role for xanthurenic acid in neurotransmission and as a neuromodulator has been proposed based on the study of distribution and transport of xanthurenic acid in rat brain (16, 17). Xanthurenic acid possesses competitive inhibitory activity of L-glutamate transport with a Kᵢ value of 190 μM (18).
These data suggest that xanthurenic acid inhibits the enzymatic activity, indicating that it could be obtained, suggesting that it could bind strongly to the NADP+ bound SPR (data not shown). Xanthurenic acid bound strongly to the NADP+ saturated SPR with a K_d of 51 ± 10 nM (n = 2), and much bound much more weakly to NADPH-bound SPR with a K_d of 9.5 ± 0.4 μM (n = 2). These data suggest that xanthurenic acid inhibits the enzymatic activity of SPR mainly by binding to the catalytic site of NADP+ -bound SPR and to a minor extent by binding to NADPH-bound SPR. This mode of inhibition is different from that of N-acetylsertotonin, which is shown to be a competitive inhibitor for pterin substrate that binds to NADPH-bound SPR (9, 26).

Is it only xanthurenic acid among various metabolites in the kynurenine pathway that inhibits SPR? To address this question, we measured IC_{50} values for human SPR of kynurenine, kynurenic acid, 8-hydroxyquinolonic acid, 3-hydroxyquinaldic acid, anthranilic acid, 3-hydroxyanthranilic acid, quinolinic acid, and picolinic acid in addition to xanthurenic acid (Fig. 1B and Table 1). The results showed that only xanthurenic acid is a potent SPR inhibitor. Finally, we measured IC_{50} values of xanthurenic acid and its analogues for mouse and rat SPR (Table 1). Xanthurenic acid also displayed high inhibitory activity against mouse and rat SPR, with IC_{50} values below 100 nM.

How do physiologically relevant concentrations of xanthurenic acid compare with the IC_{50} values measured here for SPR? It was reported that distribution of xanthurenic acid is heterogeneous in the brain of healthy rat and that its concentrations reach up to 1 μM in specific brain regions (16). It is not known whether xanthurenic acid distributes evenly or is concentrated in specific cell types in these brain regions; thus local concentrations of xanthurenic acid could be higher than 1 μM. Up-regulation of the kynurenine pathway can also result in increased xanthurenic acid concentrations. Indeed, increased metabolism of kynurenine to xanthurenic acid was demonstrated in quinolinic acid-lesioned rat striata (27). In summary, the inhibitory activity of xanthurenic acid presented here for SPR and its reported concentrations in vivo suggest that it could inhibit BH_{4} synthesis in vivo.

To gain further insights into the interaction of xanthurenic acid with human SPR, we solved the crystal structure of the enzyme with bound metabolite and NADP+ at 2.35 Å (Protein Data Bank (PDB) ID 4Z2K). The electron density clearly defines the structure and orientation of xanthurenic acid within human SPR, as demonstrated by the omit map shown at 3.5σ (Fig. 3A). Crystallographic parameters for data collection and refinement are listed in Table 2. Xanthurenic acid is bound through an extensive network of hydrogen bonds (Fig. 3B), most importantly to the catalytic residues Ser-154 and Tyr-167, and via two water molecules to Asp-254. This latter residue has been shown to be involved in substrate binding in mouse SPR (28). Further hydrogen bonds are formed between the carboxylic group and the backbone N–H of Leu-155, and between the 4-hydroxy group of xanthurenic acid and both Gln-203 and a water molecule. In addition, apolar interactions with the side chains of Trp-164, Pro-197, and Leu-219 (Fig. 3B), as well as with the nicotinamide moiety of NADP+, contribute to inhibitor binding (Fig. 3B).

The mode of binding of xanthurenic acid to human SPR shows similarities with that of other inhibitors of this enzyme, i.e. sulfasalazine (PDB ID 4J7X) and the N-acetylsertotonin analogue SPRi3 (PDB ID 4XWY) (Fig. 3B). In all cases, the inhibitors block the substrate-binding site of SPR. All three inhibitors do form hydrogen bonds with the catalytic residues Ser-154 and Tyr-167. They interact with active site residue Asp-254, although there are some noticeable differences; although the 5-hydroxy group of SPRi3 interacts directly with Asp-254 (see also Ref. 5), sulfasalazine and xanthurenic acid interact with this residue via bridging water molecules. The backbone N–H of Gly-196 plays an interesting role; in the case of xanthurenic acid and sulfasalazine, it forms a hydrogen bond with the water molecule bridging the ligand and Asp-254, whereas with SPRi3, a hydrogen bond with the 5-hydroxy group of the ligand is formed.

Hydrophobic interactions further stabilize the binding of the ligands, although to a different extent. All three inhibitors interact with Trp-164 and Pro-197. Additionally, xanthurenic acid
and sulfasalazine interact with Leu-219 and Gln-203, respectively. SPRi3 interacts with all mentioned residues and additionally with Leu-155. The nicotinamide moiety of the cofactor stacks against the pyridine ring of sulfasalazine and one of the rings of xanthurenic acid but shows only some overlap with the terminal part of the side chain of SPRi3.

The structure of SPR with xanthurenic acid allows one to rationalize the rank order of SPR inhibitory potencies of the tryptophan metabolites. Xanthurenic acid is a rigid planar molecule where all hetero-atoms are ideally positioned for hydrogen bond formation directly, or in the case of the carboxyl group via water molecules, with multiple residues of SPR. In addition, it engages in hydrophobic interactions with numerous active site residues and stacks against the nicotinamide ring of NADP\(^+\). Kynurenic acid and 8-hydroxyquininaldric acid lack one of the hydroxyl groups of xanthurenic acid; their reduced...
REPORT: Xanthurenic Acid Inhibits Sepiapterin Reductase

TABLE 2
Data collection and refinement statistics

| Data collection and processing |       |
|-------------------------------|-------|
| Beamline                      | SLS–X06DA |
| Space group                   | P6    |
| Unit cell parameters          |       |
| a, b, c (Å)                   | 143.96, 143.96, 180.74 |
| α, β, γ (°)                   | 90, 90, 120 |
| Wavelength (Å)                | 1.00000 |
| Resolution (Å)                | 47.12–2.35 (2.48–2.35) |
| Unique reflections            | 88,165 (6227) |
| Redundancy                    | 12.9 (13.3) |
| Completeness (%)              | 99.5 (100.0) |
| R_meas (%)                    | 11.0 (112.8) |
| I/σ                           | 20.0 (0.7) |
| CC1⁄2                         | 0.999 (0.816) |

Refinement and model composition

| R_meas / R_free | 0.18 (0.28) / 0.21 (0.31) |
|-----------------|---------------------------|
| No. of atoms    | 7768                       |
| Protein         | 192                        |
| NADP            | 60                         |
| H2O             | 160                        |
| SO4             | 30                         |
| EDO             | 4                          |
| Average B-factor| 49                         |
| Protein         | 50                         |
| Water           | 44                         |
| 4KL             | 37                         |
| Wilson B-factor | 57.30                      |
| r.m.s. deviations|                           |
| Bond angle (°)  | 1.4458                     |
| Bond length (Å) | 0.0197                     |

PDB ID
4Z3K

References

1. Werner, E. R., Blau, N., and Thony, B. (2011) Tetrahydrobiopterin: biochemistry and pathophysiology. Biochem. J. 438, 397–414
2. Werner, E. R., Werner-Felmayer, G., and Mayer, B. (1998) Tetrahydrobioperin, cytokines, and nitric oxide synthesis. Proc. Soc. Exp. Biol. Med. 219, 171–182
3. Costigan, M., Latremoliere, A., and Woolf, C. J. (2012) Analgesia by inhibiting tetrahydrobiopterin synthesis. Curr. Opin. Pharmacol. 12, 92–99
4. Nasser, A., Ali, S., Wilsbech, S., Bjerrum, O. J., and Møller, L. B. (2015) Intraplantar injection of tetrahydrobiopterin induces nociception in mice. Neurosci. Lett. 584, 247–252
5. Latremoliere, A., Latini, A., Andrews, N., Cronin, S. J., Fujita, M., Gorska, K., Hovius, R., Romero, C., Chuaipichai, S., Painter, M., Miracca, G., Baban, O., Remor, A. P., Duong, K., Riva, P., Barrett, L. B., Ferreiros, N., Naylor, A., Penninger, J. M., Tegeder, I., Zhong, J., Blagg, J., Channon, K. M., Johnsson, K., Costigan, M., and Woolf, C. J. (2015) Reduction of neuropathic and inflammatory pain through inhibition of the tetrahydrobiopterin pathway. Neuroph 86, 1393–1406
6. Hirakawa, H., Sawada, H., Yamahama, Y., Takeda, S., Shintaku, H., Hara, A., Mase, K., Kondo, T., and Iino, T. (2009) Expression analysis of the aldo-keto reductases involved in the novel biosynthetic pathway of tetrahydrobiopterin in human and mouse tissues. J. Biochem. 146, 51–60
7. Chidley, C., Haruki, H., Pedersen, M. G., Muller, E., and Johnsson, K. (2011) A yeast-based screen reveals that sulfaalazine inhibits tetrahydrobiopterin biosynthesis. Nat. Chem. Biol. 7, 375–383
8. Haruki, H., Pedersen, M. G., Gorska, K. I., Pojer, F., and Johnsson, K. (2013) Tetrahydrobiopterin biosynthesis as an off-target of sulfa drugs.
9. Katoh, S., Sueoka, T., and Yamada, S. (1982) Direct inhibition of brain serotonin uptake by a catecholamine and an indoleamine. Biochem. Biophys. Res. Comm. 105, 75–81

10. Smith, G. K., Duch, D. S., Edelstein, M. P., and Bigham, E. C. (1992) New inhibitors of serotonin reuptake. Lack of an effect of intracellular tetrahydrobiopterin depletion upon in vitro proliferation of two human cell lines. J. Biol. Chem. 267, 5599–5607

11. Jégou, E., Lovenberg, W., and Sjoerdsma, A. (1967) Tryptophan hydroxylase inhibition: the mechanism by which p-chlorophenylalanine depletes rat brain serotonin. Mol. Pharmacol. 3, 274–278

12. Tong, J. H., and Kaufman, S. (1975) Tryptophan hydroxylase. Purification and some properties of the enzyme from rabbit hindbrain. J. Biol. Chem. 250, 4152–4158

13. Han, Q., Beerntsen, B. T., and Li, J. (2007) The tryptophan oxidation pathway in mosquitoes with emphasis on xanthurenic acid biosynthesis. J. Insect Physiol. 53, 254–263

14. Colín-González, A. L., Maldonado, P. D., and Santamaría, A. (2013) 3-Hydroxykynurenine: an intriguing molecule exerting dual actions in the central nervous system. Neurotoxicology 34, 189–204

15. Reyes Ocampo, J., Lugo Huitrón, R., González-Esquivel, D., Ugalde-Muñiz, P., Jiménez-Anguiano, A., Pineda, B., Pedraza-Chaverri, J., Ríos, C., and Pérez de la Cruz, V. (2014) Kynurenines with neuroactive and redox properties: relevance to aging and brain diseases. Oxid. Med. Cell. Longev. 2014, 646909

16. Gobaille, S., Kemmel, V., Brumaru, D., Dugave, C., Aunis, D., and Maitre, M. (2008) Xanthurenic acid distribution, transport, accumulation and release in the rat brain. J. Neurochem. 105, 982–993

17. Taleb, O., Maammar, M., Brumaru, D., Bourguignon, J.-J., Schmitt, M., Klein, C., Kemmel, V., Maitre, M., and Mensah-Nyagan, A. G. (2012) Xanthurenic acid binds to neuronal G-protein-coupled receptors that secondarily activate cationic channels in the cell line NCB-20. PLoS ONE 7, e48553

18. Bartlett, R. D., Esslinger, C. S., Thompson, C. M., and Bridges, R. J. (1998) Substituted quinolines as inhibitors of l-glutamate transport into synaptic vesicles. Neuropharmacology 37, 839–846

19. Schimke, R. T., Sweeney, E. W., and Berlin, C. M. (1965) The roles of synthesis and degradation in the control of rat liver tryptophan pyrrole. J. Biol. Chem. 240, 322–331

20. Patnaik, S. K., and Sarangi, S. (1980) Age-related response of tryptophan pyrrole to 1β-estradiol in the liver of female Rats. J. Biochem. 87, 1249–1252

21. Yamazaki, F., Kuroiwa, T., Takikawa, O., and Kido, R. (1985) Human indoleamine 2,3-dioxygenase: its tissue distribution, and characterization of the placental enzyme. Biochem. J. 230, 635–638

22. Takikawa, O., Yoshida, R., Kido, R., and Hayashi, O. (1986) Tryptophan degradation in mice initiated by indoleamine 2,3-dioxygenase. J. Biol. Chem. 261, 3648–3653

23. Munn, D. H., and Mellor, A. L. (2013) Indoleamine 2,3 dioxygenase and metabolic control of immune responses. Trends in Immunology 34, 137–143

24. Platten, M., Wick, W., and Van den Eynde, B. J. (2012) Tryptophan catabolism in cancer: beyond IDO and tryptophan depletion. Cancer Res. 72, 5435–5440

25. Schwarz, R., Bruno, J. P., Muchowski, P. J., Wu, H.-Q. (2012) Kynurenines in the mammalian brain: when physiology meets pathology. Nat. Rev. Neurosci. 13, 465–477

26. Yang, S., Jan, Y.-H., Gray, J. P., Mishin, V., Heck, D. E., Laskin, D. L., and Laskin, J. D. (2013) Tryptophan reductase mediates chemical redox cycling in lung epithelial cells. J. Biol. Chem. 288, 19221–19237

27. Guidetti, P., Eastman, C. L., and Schwarzw, R. (1995) Metabolism of [5-3H]kynurenine in the rat brain in vivo: evidence for the existence of a functional kynurenine pathway. J. Neurochem. 65, 2621–2632

28. Auerbach, G., Herrmann, A., Gültlich, M., Fischer, M., Jacob, U., Bacher, A., and Huber, R. (1997) The 1.25 Å crystal structure of tryptophan reductase reveals its binding mode to perin and brain neurotransmitters. EMBO J. 16, 7219–7230

29. Friedman, J., Roze, E., Abdenur, J. E., Chang, R., Gasperini, S., Saletti, V., Wali, G. M., Eiroa, H., Neville, B., Felice, A., Parascandalo, R., Zeferiu, D. I., Arrabal-Fernandez, L., Dill, P., Eichler, F. S., Echenne, B., Gutierrez-Solana, L. G., Hoffmann, G. F., Hyland, K., Kusmierska, K., Tijssen, M. A. J., Lutz, T., Mazzuca, M., Penzien, J., Poll-The, B. T., Sykut-Cegiel ska, J., Zysmanska, K., Thöny, B., and Blau, N. (2012) Tryptophan reductase deficiency: a treatable mimic of cerebral palsy. Ann. Neurol. 71, 520–530

30. England, J. M., and Coles, M. (1972) Effect of co-trimoxazole on phenylalanine metabolism in man. Lancet 2, 1341–1343

31. Andrews, T. M., Purkiss, P., Chalmers, R. A., and Watts, R. W. E. (1976) Effect of cotrimoxazole on the response to phenylalanine loading in man. Clin. Chim. Acta 65, 123–130

32. Raison, C. L., Dantzer, R., Kelley, K. W., Lawson, M. A., Woolwine, B. J., Vogt, G., Spivey, J. R., Saito, K., and Miller, A. H. (2010) CSF concentrations of brain tryptophan and kynurenines during immune stimulation with IFN-α: relationship to CNS immune responses and depression. Mol. Psychiatry 15, 393–403

33. Dantzer, R., O’Connor, J. C., Lawson, M. A., and Kelley, K. W. (2011) Inflammation-associated depression: From serotonin to kynurenine. Psychoneuroendocrinology 36, 426–436

34. Felger, J. C., Li, L., Marvar, P. J., Woolwine, B. J., Kozin, G., Spivey, J. R., Saito, K., and Miller, A. H. (2010) Tyrosine metabolism during interferon-α administration: association with fatigue and CSF dopamine concentrations. Brain Behav. Immun. 31, 153–160

35. Dantzer, J. F., Hernandez-Castillo, C. R., and Miller, A. H. (2013) Levodopa reverses cytokine-induced reductions in striatal dopamine release. Int. J. Neuropsychopharmacol. 18, pyu084

36. Zoller, H., Schloegl, A., Schroeksnadel, S., Vogel, W., and Fuchs, D. (2012) Interferon-α therapy in patients with hepatitis C virus infection increases plasma phenylalanine and the phenylalanine to tyrosine ratio. J. Interferon Cytokine Res. 32, 216–220

37. Klassen, P., Fürst, P., Schulz, C., Mazariogios, M., and Solomon, N. W. (2001) Plasma free amino acid concentrations in healthy Guatemalan adults and in patients with classic dengue. Am. J. Clin. Nutr. 73, 647–652

38. Lopansri, B. K., Anstey, N. M., Stoddard, G. J., Mwaikambo, E. D., Boutlis, C. S., Tjitra, E., Maniboey, H., Hobbs, M. R., Levesque, M. C., Weinberg, J. B., and Granger, D. L. (2006) Elevated plasma phenylalanine in severe malaria and implications for pathophysiology of neurological complications. Infect. Immun. 74, 3355–3359

39. Spener-Unterweger, B., Kohl, C., and Fuchs, D. (2014) Immune changes and neurotransmitters: Possible interactions in depression? Progress in Neuro-Psychopharmacology and Biological Psychiatry 48, 268–276