Investigation of Proposed Activity of Clarithromycin at GABA_A Receptors Using [¹¹C]Flumazenil PET

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S Supporting Information

ABSTRACT: Clarithromycin is a potential treatment for hypersomnia acting through proposed negative allosteric modulation of GABA_A receptors. We were interested whether this therapeutic benefit might extend to Parkinson's disease (PD) patients because GABAergic neurotransmission is implicated in postural control. Prior to initiating clinical studies in PD patients, we wished to better understand clarithromycin's mechanism of action. In this work we investigated whether the proposed activity of clarithromycin at the GABA_A receptor is associated with the benzodiazepine binding site using *in vivo* [¹¹C]flumazenil positron emission tomography (PET) in primates and *ex vivo* [³H]flumazenil autoradiography in rat brain. While the studies demonstrate that clarithromycin does not change the K_d of FMZ, nor does it competitively displace FMZ, there is preliminary evidence from the primate PET imaging studies that clarithromycin delays dissociation and washout of flumazenil from the primate brain in a dose-dependent fashion. These findings would be consistent with the proposed GABA_A allosteric modulator function of clarithromycin. While the results are only preliminary, further investigation of the interaction of clarithromycin with GABA receptors and/or GABAergic medications is warranted, and therapeutic applications of clarithromycin alone or in combination with flumazenil, to treat hyper-GABAergic status in PD at minimally effective doses, should also be pursued.

KEYWORDS: Hypersomnia, Parkinson's disease, positron emission tomography, [¹¹C]FMZ

Central nervous system (CNS) hypersomnias are a group of disorders (e.g., narcolepsy, idiopathic hypersomnia) that causes excessive daytime sleepiness in patients, despite enough quality nocturnal sleep.¹ Recent work from Rye and co-workers suggests that many hypersomnia patients have an endogenous, positive allosteric modulator of γ -aminobutyric acid-A (GABA_A) receptors in their cerebrospinal fluid.² Given the known involvement of GABA in promoting sleep,³ it follows that dysfunction of the GABA system could play a role in hypersomnias, and this is supported in further work by the Rye group, who demonstrated improved vigilance in hypersomnia patients treated with a combination of sublingual and transdermal flumazenil (FMZ, Figure 1),² an established negative allosteric modulator of GABA_A receptors.⁴ However, FMZ is only approved for intravenous use and is therefore a less favorable treatment for hypersomnia patients than an oral drug would be. Therefore, orally administered negative

Figure 1. Flumazenil (FMZ).

allosteric moderators/antagonists of GABA_A receptors have been explored as alternative treatments.^{5,6}

Clarithromycin (Figure 2), a macrolide antibiotic approved in both oral and intravenous forms, has a range of neurotoxic side effects, including excitation, mania, psychosis, insomnia, and nonconvulsive status epilepticus.^{7–10} Although the

Received: November 13, 2015

Accepted: June 1, 2016

Published: June 1, 2016

Figure 2. Clarithromycin.

mechanism of action underlying these CNS side effects is unknown,⁷ clarithromycin is purported to be a negative allosteric modulator of GABA_A receptors from *in vitro* whole-cell patch clamp experiments, albeit with low affinity (18% and 45% inhibition at 3 and ~300 μ M, respectively).^{11,12} Notably, one patient being treated for hypersomnia with FMZ was also prescribed clarithromycin to manage acute bronchitis, and the combination of therapies resulted in a period of prolonged insomnia, which only resolved upon discontinuation of clarithromycin.^{2,5} These findings suggest not only pronounced GABAergic effects but also that clarithromycin crosses the blood–brain barrier (BBB).¹³ Building on these findings, clarithromycin is being tested as a potential treatment for hypersomnia, and improvement in daytime sleepiness has been reported in initial clinical studies for patients on clarithromycin 500 mg twice per day vs placebo.^{5,6}

These results were of particular interest to us because of our long-standing research interests in understanding the mechanisms underlying Parkinson's disease (PD) and our extensive work managing patients with PD. Axial motor impairments represent a significant cause of disability in PD, which is mainly explained by lack of efficacy of dopaminergic treatments on these symptoms.^{14,15} Treatment approaches to reduce balance problems and falls has been recently identified as the number 1 research priority in PD.¹⁶ Cholinergic system dysfunction has been implicated in some components of postural instability and gait difficulties (PIGD) in PD, in particular falls and sensory processing during postural control, but not with overall severity of axial motor impairments.¹⁷ Postural control and gait functions are mediated by widespread neural networks that cannot be captured by a simplistic model of single neurotransmitter system changes. There is increasing interest in dysfunction of colocalized neurotransmitter functions in PD to better understand the complexity of the multisystem nature of neurodegeneration. GABAergic neurotransmission has been implicated in postural control because of the well-known increased fall risk in elderly taking benzodiazepine drugs.¹⁸ In this respect, it is of interest that the main output structures of the basal ganglia (the globus pallidus internus and the substantia nigra pars reticularis) provide tonic GABAergic inhibitory tone over the brainstem structures that control gait and balance (such as the pedunculopontine nucleus) and the motor thalamus, effectively constraining information flow in the spinal cord and cortex, respectively.¹⁹ There is now abundant evidence to demonstrate that excessive stimulation of the output structures of the basal ganglia leads to increased GABAergic inhibition of the brainstem and thalamic structures that control gait, which ultimately manifests with gait and balance problems in PD.^{19–21} Excessive GABAergic activity in the pons has been confirmed by *in vivo* MR spectroscopy in patients with PD.²² As the pedunculopontine nucleus is the major modulator of the thalamus and its related central role of postural control in PD,¹⁷ the presence of overactive GABAergic

inhibition in the brainstem in PD would auger novel pharmacological approaches to block excessive GABAergic inhibition in PD. In particular, intravenous FMZ has been shown to rapidly improve motor impairments in PD patients.²³ Following the described hypersomnia findings, we were curious whether oral clarithromycin might also be a more practical treatment than intravenous FMZ in our PD patient population.

Before initiating clinical studies in PD, however, we wished to have a better understanding of clarithromycin's mechanism of action. Given that clarithromycin has been shown to reverse hypersomnia like FMZ, a GABA antagonist that binds to the benzodiazepine (BZ) binding site of the GABA_A receptor,⁴ the focus of this initial study was to determine whether clarithromycin acts through the same mechanism. In particular, we wished to evaluate clarithromycin binding to the BZ site on the GABA_A receptor and estimate GABA_A receptor occupancy by clarithromycin at differing doses. We reasoned the former could be accomplished through *in vitro* binding affinity experiments using competition assays with [³H]FMZ in tissue, while the latter should be achievable through functional positron emission tomography (PET) imaging in nonhuman primates using [¹¹C]FMZ, and in this letter, we report our findings from both sets of experiments.

We initially undertook binding affinity experiments through competition assays between clarithromycin and [³H]FMZ. Saturation curves for [³H]FMZ in rat brain were determined, giving a K_d of 0.66 ± 0.12 nM and a B_{max} of 0.75 ± 0.06 fmol/ μ g ($n = 2$). These values are consistent with literature values of K_d and B_{max} for FMZ (human: $K_d = 12$ nM, $B_{max} = 0.12 \pm 0.014$ fmol/ μ g;²⁴ Baboon: $K_d = 15.8 \pm 2.2$ nM, $B_{max} = 0.07 \pm 0.015$ fmol/ μ g;²⁵ Rat: $K_d = 1.35 \pm 0.316$ nM, $B_{max} = 0.638 \pm 0.099$ fmol/ μ g²⁶). Competition studies in rat brain sections were then done with concentrations of clarithromycin ranging from 10 nM–10 μ M (Figure 3 and Supporting Information, SI).

Figure 3. Representative autoradiography images showing high specific binding of [³H]FMZ in the cortex of the rat brain despite increasing concentrations (10 nM, 100 nM, 1 μ M, and 10 μ M) of clarithromycin.

Concentrations >10 μ M were not tested as we reasoned that such concentrations could not realistically be achieved *in vivo* (clarithromycin is approved to be given to adults in doses ranging from 250–500 mg, twice per day; steady-state peak serum concentrations are 1.3–2.0 and 2.7–4.0 μ M after multiple 250 and 500 mg doses, respectively;²⁷ patients in Rye's hypersomnia study received 500 mg, twice per day over a two-week period, although certain subjects were increased to 1000

mg, twice per day, if the lower doses were not sufficient to alleviate symptoms of hypersomnia^{5,6}). However, we saw no displacement of [³H]FMZ, even at the 10 μ M concentration of clarithromycin (Figure 3), indicating that the K_i of clarithromycin for the BZ binding site on GABA_A receptors is >10 μ M.

Since there was no evidence *in vitro* of direct clarithromycin binding to the BZ site on the GABA_A receptor complex, we next wanted to explore whether clarithromycin was modulating FMZ binding *in vivo*. We conducted functional *in vivo* PET imaging studies in nonhuman primates ($n = 2$) using [¹¹C]FMZ, and regional tissue time-activity curves (TACs) were generated for the cortex. Representative images and TACs for one of the monkeys are shown in Figures 4 and 5,

Figure 4. Representative summed coronal [¹¹C]FMZ PET images from monkey #1 (50–90 min after *i.v.* injection of the radiotracer) of control animal (A), after displacement with 0.5 mg/kg FMZ (B), and following treatment with 3.5 (C) or 7 mg/kg (D) clarithromycin.

Figure 5. Representative cortical time-radioactivity curves for monkey #1: control study (■), after displacement with 0.5 mg/kg FMZ (□), and following treatment with 3.5 (●) or 7 mg/kg (◆) clarithromycin.

respectively. A baseline study in the monkey brain (Figure 4a) showed a time-dependent regional brain distribution that correlated with the known distribution of GABA_A receptors, with the highest specific binding in the cortex. In a positive control experiment, nearly complete washout of radioactivity from all brain regions occurred after injection of 0.5 mg/kg unlabeled FMZ (Figure 4b), consistent with previous results for

[¹¹C]FMZ or [¹⁸F]FMZ using comparative displacing doses of FMZ.^{28,29}

We next investigated displacement of [¹¹C]FMZ following *i.v.* injection with clarithromycin in nonhuman primates ($n = 2$). Given the numerous side effects associated with clarithromycin (*vide supra*), as well as increased bioavailability expected from an *i.v.* dose, we initially considered the lower 250 mg human dose as a starting point; reasoning that 250 mg administered to a 70 kg human corresponds to 3.5 mg/kg, we selected this dose for the first nonhuman primate blocking study.³⁰ At 40 min postinjection of [¹¹C]FMZ, the monkey received an infusion of clarithromycin (0.07 mg/kg/min) for the next 50 min (corresponding to a total dose of 3.5 mg/kg by the conclusion of the 90 min PET scan). However, analysis of the PET image (Figure 4c) indicated no obvious displacement of [¹¹C]FMZ in the time frame of the PET scan. Doubling the dose to 7 mg/kg total infusion similarly did not displace [¹¹C]FMZ (Figure 4d). In light of the *in vitro* binding data described above, where no displacement of [³H]FMZ was apparent even at 10 μ M concentrations of clarithromycin, we elected not to increase the primate dose any higher than 7 mg/kg, concluding that in this series of studies there is no evidence that clarithromycin displaces FMZ from the benzodiazepine binding site of the GABA_A receptor *in vitro* or *in vivo*.

The TACs for the imaging studies showed that after *i.v.* injection of [¹¹C]FMZ, cortical uptake of radioactivity was rapid (Figure 5). Injection of a high dose of unlabeled FMZ (0.5 mg/kg) at 40 min initiated a rapid washout of radioactivity, with nearly complete clearance by the end of the PET study. The TACs also confirm no displacement of [¹¹C]FMZ by clarithromycin. Intriguingly, however, the TACs of the two clarithromycin studies do not exactly resemble the TAC of the baseline study. There is evidence that clarithromycin delays dissociation and washout of [¹¹C]FMZ in a dose-dependent fashion. The effect was more pronounced in one of the monkeys than the other, although the trend was apparent in both animals. To examine this finding further, Logan plot analyses were undertaken (see SI).³¹ The pons was used as a reference region (as previously described³²) to estimate early (10–40 min postinjection of [¹¹C]FMZ) and late (50–90 min postinjection of [¹¹C]FMZ corresponding to duration of clarithromycin infusion) distribution volume ratios (DVRs) with the intent of understanding [¹¹C]FMZ kinetics before and after pharmacological challenge with clarithromycin.³³ Logan analysis gave mixed results. Increases in late DVRs in the clarithromycin blocking studies confirmed slower washout in monkey #1 (late DVR/early DVR = 121%, 131%, and 186% for baseline, 3.5 mg/kg clarithromycin, and 7 mg/kg clarithromycin, respectively). In monkey #2 there were similar differences in the TACs, but early and late DVR values were similar (late DVR/early DVR = 115%, 108%, and 89% for baseline, 3.5 mg/kg clarithromycin, and 7 mg/kg clarithromycin, respectively), possibly because of the higher and more variable late uptake of [¹¹C]FMZ in the reference region during the 7 mg/kg clarithromycin study.

One possible explanation for these findings is that clarithromycin does indeed enter the CNS and interacts with the GABA_A receptor. Given that $K_d = K_{off}/K_{on}$, if clarithromycin were to interact with the GABA_A receptor in a way that did not displace FMZ, but rather decreased the K_{off} , this would decrease the K_d of FMZ and could potentially enhance its CNS effects. Such a pharmacological outcome could lead to the synergistic effects of coadministered clarithromycin and FMZ observed in

preliminary clinical studies.^{2,5} To evaluate the potential effects of clarithromycin on dissociation of FMZ from the GABA_A receptor, we repeated the rat brain saturation curves for [³H]FMZ to determine K_d in the presence of 10 μ M clarithromycin and obtained a value of 0.64 ± 0.11 nM, a nonsignificant decrease from control values. In a second study, rat brain sections were incubated with [³H]FMZ, in the presence and absence of 10 μ M clarithromycin, for 30 min to reach equilibrium. Ten micromolar FMZ was then added, and brain sections were removed and analyzed (by phosphorimager) at various time points (0, 2, 4, 8, and 16 min) after addition of FMZ. There was no difference in displacement of [³H]FMZ from the control brain sections and those incubated with clarithromycin (Figure S3).

Clarithromycin could also alter CNS pharmacokinetics of FMZ through other mechanisms such as slowing down FMZ metabolism. FMZ is probably metabolically demethylated by a cytochrome P450 (CYP), and possibilities include CYP3A.³⁴ Clarithromycin is a known CYP3A inhibitor that has been shown to slow metabolism, reduce clearance, and increase the area under the curve of orally administered benzodiazepines such as midazolam,³⁵ and it is feasible that it is playing a similar role in CNS pharmacokinetics of [¹¹C]FMZ in the primate imaging studies. In such a scenario, we would expect higher levels of [¹¹C]FMZ in the reference region (pons) in treated monkeys, and there is some evidence of this in the time-radioactivity curves for all of the monkey studies (see pons TACs in SI). However, the results are noisy, and further investigation of tracer metabolism in the presence and absence of clarithromycin will be explored before definitive conclusions are made.

In summary, the use of clarithromycin to manage symptoms of hypersomnia in a clinical setting is increasingly well documented, with negative allosteric modulation of GABA_A receptors being the proposed mode of action. In this work, *in vitro* binding assays demonstrated clarithromycin does not change the K_d of [³H]FMZ, nor does clarithromycin competitively displace [³H]FMZ at concentrations up to 10 μ M. Similarly, *i.v.* doses of clarithromycin up to 7 mg/kg were insufficient to displace cortical [¹¹C]FMZ binding in nonhuman primate PET studies. These results suggest it is unlikely that CNS effects of clarithromycin are due to binding at the BZ site of the GABA_A receptor. However, we present preliminary evidence that clarithromycin may delay dissociation of [¹¹C]FMZ in a dose-dependent fashion. One possible explanation for this finding includes the proposed GABA_A negative allosteric modulator function of clarithromycin. Alternatively, clarithromycin could change FMZ pharmacokinetics through other means such as reducing blood flow, increasing free fraction of the radiotracer, or altering tracer metabolism. Further investigation of these alternate explanations is ongoing and will be reported in due course.

While results are only preliminary at this stage, when combined with known clinical efficacy of clarithromycin in managing hypersomnia, further investigation of the interaction of clarithromycin with GABA receptors and/or GABAergic medications seems warranted. Screening of clarithromycin for activity at other GABA binding sites and/or receptors,^{36,37} with the aim of further understanding the origin of its wide-ranging CNS effects, as well as evaluation of BBB permeability, is also recommended. Efforts in this direction are underway in our laboratory. Given the known potential of neurotoxicity of clarithromycin at higher dose levels, therapeutic applications of

this drug alone or in combination with flumazenil to treat hyper-GABAergic status in PD at minimally effective doses should also be pursued.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmchemlett.5b00435.

Experimental details for radiochemical syntheses and quality control; protocols for primate PET imaging studies; procedures for *in vitro* binding affinity and autoradiography studies (PDF)

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Author Contributions

The manuscript was written through contributions of all authors, and all authors have given approval to the final version of the manuscript.

Funding

Financial support of this work from the University of Michigan Department of Radiology is gratefully acknowledged.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank the staff of the University of Michigan PET Center for assistance with the synthesis and quality control of [¹¹C]FMZ.

■ ABBREVIATIONS

BBB, blood–brain barrier; BZ, benzodiazepine; CNS, central nervous system; CYP, cytochrome P450; DVR, distribution volume ratio; FMZ, flumazenil; GABA, γ -aminobutyric acid; *i.v.*, intravenous; PD, Parkinson's disease; PET, positron emission tomography; ROI, region of interest; TAC, time–activity curve

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