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Commentary

The challenge of drug discovery of a GPCR target: Analysis of preclinical pharmacology of histamine H_3 antagonists/inverse agonists

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ABSTRACT

Although the histamine H_3 receptor was identified pharmacologically in 1983, and despite widespread pharmaceutical interest in the target, no compound interacting specifically with this site has undergone successful clinical examination to develop the necessary proof-of-concept data. Therefore, clinical knowledge of the therapeutic potential of H_3 receptor antagonists in neuropsychiatric diseases, in metabolic diseases or in sleep disorders has yet to determine if the preclinical data that show broad efficacy in animal models of the aforementioned states are relevant to current unmet medical needs. H_3 receptors are complex, with species-related sequence differences that impact pharmacological responses. The receptors have a complex gene organization that provides opportunity for multiple slice isoforms, most of which remain poorly characterized even within a species. H_3 receptors are constitutively active, although the extent of this could vary either between species and/or receptor splice isoforms, both of which may provide opportunity for preferential coupling to different G-proteins. Thus, it is not surprising that the pharmacological effects of known H_3 ligands are complex and diverse, since these agents may act both as agonists and antagonists in different systems. Moreover, other compounds show inverse agonism in some models but neutral antagonist activity in others. Some of this diversity may be related to different ligand-dependent receptor activation states or to the effects of key amino acids important for ligand recognition. This commentary provides an overview of these complexities as applied to the H_3 receptor and the challenges these intricacies create for drug discovery.

1. Introduction

Drugs targeting G-protein coupled receptors (GPCRs) represent one of the most successful classes of pharmaceutical remedies known. In recent years, it was estimated that as many as 50% of available drugs act directly via stimulating or blocking GPCRs [1]. Among such drugs are the antiallergy medications, such as the classical ''antihistamines'' (e.g., diphenhydramine), non-sedating second-generation compounds (astemizole, terfenadine, loratidine), or third-generation compounds (norastemizole, fexofenadine, desloratidine) and antiulcer medications (cimetidine, ranitidine, famotidine). These compounds are antagonists of H_1 and H_2 receptors, respectively. Since the 1983 discovery of the H_3 receptor [2] and the 2000

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identification of the H_4 receptor [3], it has become clear that histamine (HA) has a total of four known GPCR targets, and a number of pharmaceutical companies are actively pursuing novel compounds targeting these newest histamine receptors for various therapeutic indications.

From studies of the H_3 receptor, a number of novel findings have emerged, suggesting that the H_3 receptor has many levels of complexity, many of which have direct implications for the process of drug discovery. Novel compounds have been identified that interact with H_3 receptors, arising from diverse research programs. Yet, to date, no compounds have successfully met the challenges of the drug development process to allow sufficient clinical evaluation and, thereby, enable proof-of-concept for the various therapeutic indications that have been proposed for H_3 receptor agonists and antagonists. This commentary focuses on how aspects of H_3 receptor complexity have led to advances in our knowledge of receptor pharmacology and the advancement of compounds with ever improving drug-like properties toward eventual clinical use, with most examples taken from the experiences of colleagues at Abbott Laboratories.

2. Species-dependent H_3 receptor pharmacology: implications for H_3 antagonist drug discovery

Between its discovery in 1983 [2] and 1999, attempts to clone the H_3 receptor were unsuccessful until Lovenberg et al. correctly identified and functionally characterized the human H_3 receptor [4]. In retrospect, homology-searching strategies, as had been performed successfully for many other biogenic amine receptors, failed because the H_3 receptor gene and protein are so distinct from the previously cloned H_1 and H_2 receptors [5]. The rapid success (within 12 months) by multiple investigators to obtain the H_4 receptor sequence by homology strategies underscores the unpredictability of de-orphanizing GPCRs. In fact, the H_3 receptor was first sequenced by investigators at Millennium Pharmaceuticals [6], thinking it to be a muscarinic receptor based on its affinity for acetylcholine. Building upon the sequence of the human receptor, Lovenberg and others cloned the rat, mouse, monkey, Syrian hamster, and guinea-pig receptors, and a partial canine sequence was also obtained in our labs (for review, see Ref. [7]). More importantly, significant pharmacological differences were shown for the affinity of a number of prototype H_3 receptor antagonists when comparing the rat and the human H_3 receptor [8].

This finding had a direct and immediate impact on the drug discovery process within our laboratories. Our studies had begun with a high-throughput screen of our compound collection, using a radioligand binding assay and rat brain homogenates. We uncovered a number of hits of various chemical structures. We had made a decision to avoid imidazole-based compounds, unlike all the prototypic compounds available at the time (thioperamide, GT-2016 [5-cyclohexyl-1-(4-imidazol-4-ylpiperidyl)pentan-1-one], etc.; Fig. 1), because of the known liabilities of some of these compounds and of imidazoles, in general (for review, see Ref. [9]). One of our hits was A-923 (4-[3-(4-hexanoyl-phenoxy)-propyl]-piperazine-1-carboxylic acid ethyl ester; Fig. 1), which had nanomolar affinity for the rat H_3 receptor, but was not particularly H_3 selective nor a bioavailable compound. Medicinal chemists synthesized analogs that retained or improved upon the potency at the H_3 receptor, were much more selective, and had favorable pharmacokinetic properties (e.g., A-304121 (4-(3-((2R)-2-aminopropanoyl-1-piperazinyl)-propoxy)phenyl)(-cyclopropyl) methanone; Fig. 1; [10]). Interestingly, initial interest in this compound was diminished by the fact that the first salt prepared, a trifluoro-acetic acid salt, had about 10-fold lower affinity for the receptor than A-923. However, preparation of the free base or of several tartrate salts showed that A-304121 had affinity of about 1 nM at the rat H_3 receptor and excellent selectivity, including low affinity at more than 75 other receptors tested either at CEREP or internally. Since this finding, we have avoided trifluoro-acetic acid salts of compounds, which cause an artifactual loss of affinity in H_3 receptor binding assays. To validate that the compound was an antagonist, we turned to a guinea-pig ileum assay, in which H_3 receptor agonists diminish a field-stimulated twitch response (acetylcholine-release-mediated), an effect reversed by H_3 antagonists [11]. This assay provided our first clue (in retrospect) that something was amiss. The potency of A-304121 in this functional assay was much less than expected, providing a pA_2 value of only 6.27 (equivalent to 537 nM). At the same time, we obtained human brain homogenates, finding, to our dismay that the affinity of A-304121 was over 800 nM. These findings suggested species-dependent receptor heterogeneity, which is amply described in receptor pharmacology literature, and was already found comparing the mouse to the guinea-pig H_3 receptors [12]. However, the 800-fold difference between the rat and human homogenate binding potencies was perhaps the most discrepant in pharmacological literature, to the best of our knowledge.

When we realized that the H_3 receptor had been sequenced, we were able to clone the same receptor as that reported by Millennium. Transient expression studies confirmed the disturbing news that our lead compound was nearly 1000-fold less potent at the human receptor than at the rat receptor, making it essentially non-selective with respect to many other receptors and too weak to consider as a useful lead compound for human therapy. In addition, ongoing toxicity tests showed the compound to have effects that were attributable to its dibasic amine and cationic amphiphilic nature, namely phospholipidosis, a condition of altered phospholipids metabolism that generally results in excessive intracellular deposition of phospholipids [13]. Clinical consequences of phospholipidosis have been reported, for example, pulmonary dysfunction, with fluoxetine [14].

Analogs of A-304121 were prepared [15] that eliminated the basicity of one of the amines, for example, in A-317920 (N-((1R)-2-(4-(3-(4-(cyclopropylcarbonyl) phenoxy)-propyl)-1-piperazinyl)-1-methyl-2-oxoethyl-)-2-furamide; Fig. 1). This compound was free of the toxicological effects inherent in A-304121, thus, validating the hazards of dibasic cationic amphiphiles as potential drugs, and had favorable pharmacokinetic properties, but still suffered from poor affinity for the human H_3 receptor (63 nM, K_i), precluding its consideration as a development candidate. In fact, this series of compounds provided very few compounds with balanced affinity across species of receptor. An example, A-320436 (furan-2-carboxylic

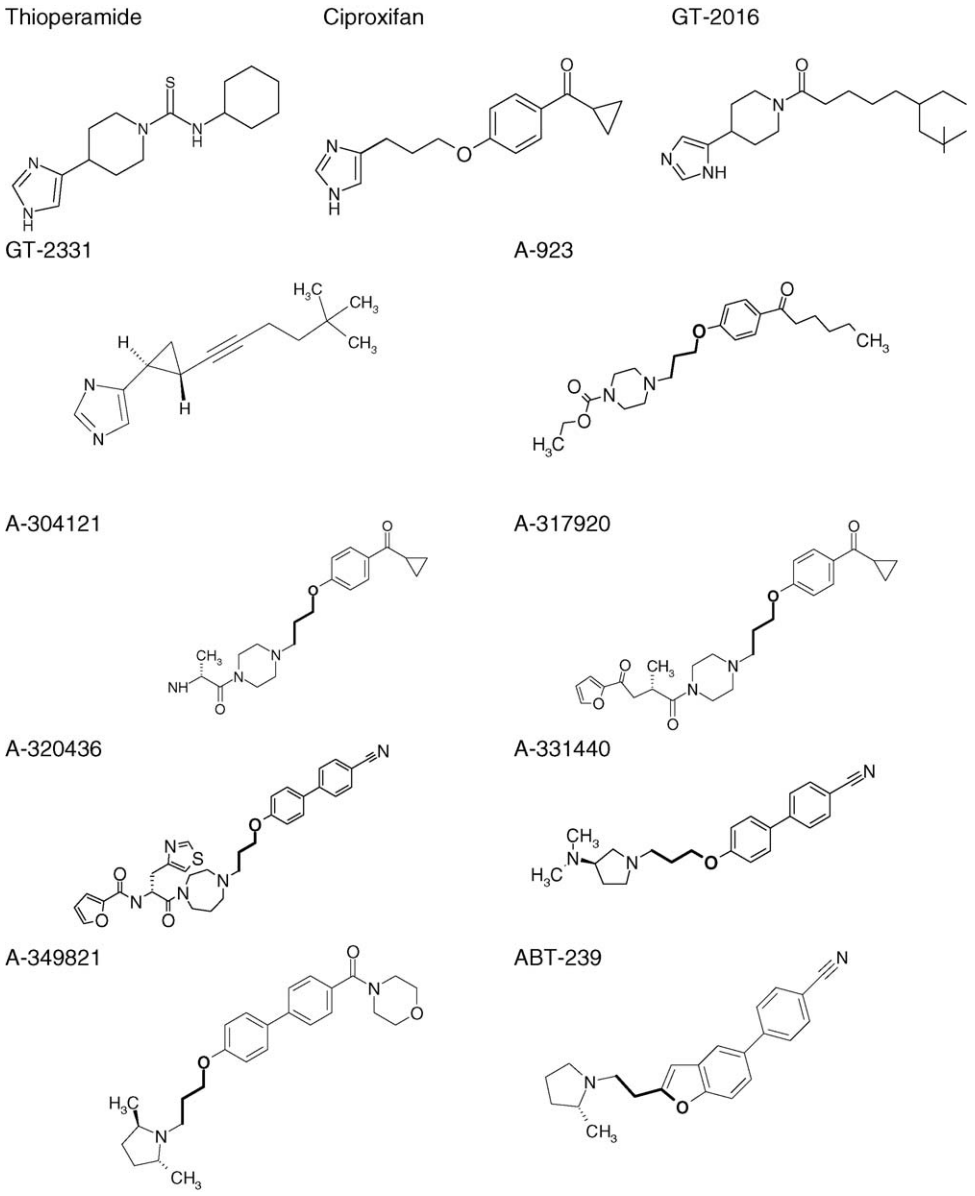

Fig. 1 – Chemical structures of reference and Abbott investigational H_3 receptor antagonists/inverse agonists. The propyloxy-linking group common to many compounds is highlighted on ciproxifan and the Abbott compounds.

acid (2-{4-[3-(4′-cyano-biphenyl-4-yloxy)-propyl]-[1,4] diazepan-1-yl}-2-oxo-1-thiazol-4-ylmethyl-ethyl)-amide; Fig. 1), while having nanomolar affinity for both species [16], suffered from very poor pharmacokinetic properties. On the other hand, these compounds had value as tools to help define the pharmacological characteristics of H_3 receptors from various species.

Once the sequence of the rat and human H_3 receptors became known and indications of potency differences for several known ligands were described [8], several groups sought the source of these differences. The first to publish was Ligneau et al. [17] who performed point mutations on the rat H_3 receptor, changing residues 119 and 122 either singly or simultaneously from A(119) and V(122) of the rat sequence to T(119) and A(122) of the human H_3 receptor, noting a decrease in the affinity of ciproxifan (Fig. 1) by approximately 5–18-fold, respectively. In simultaneous studies, we began with a molecular modeling approach to compare the rat and human protein sequences, also identifying these two amino acids in transmembrane spanning domain 3 as the most likely (of the 16 amino acids that differed between these two species) to play a role in a binding domain and potency differences [18]. Of course, the hypothesis to focus on these regions of the H_3 receptor was largely derived from analysis of GPCR structure and function using techniques, such as those pioneered by Strader et al. [19]. We began with a chimeric receptor (human sequence 1–144 [through transmembrane domain 3], rat sequence 145–445) confirming that the chimera retained the human-like affinity for compounds like A-304121, then singly or doubly mutating the human gene to convert the expressed peptide into T119A and/or A122V (i.e., human to rat). Such changes dramatically increased the affinity of the receptor for

compounds like A-304121. Interestingly, single mutations, especially of A122V provided affinities for compounds like A-304121 that were intermediate between the human and rat, more analogous to those of the guinea-pig (as revealed either in binding assays or from the guinea-pig ileum assay). The role of these amino acids was further validated upon the cloning of the guinea-pig [20] or dog (partial sequence, see Ref. [7]) receptor that had the sequence 122V (rat-like), 119T (human-like) and for which compounds like A-304121 had affinities intermediate between their high potencies for rat H_3 and their lower affinities for human H_3 receptors. The use of tool compounds having a far greater disparity in their affinities across species was extremely useful in rationalizing the potency differences observed [11] and in suggesting the need for another round of high-throughput screening to identify compounds with more equal affinities at the human and rat receptors.

While the high-throughput screening campaign using H_3 receptors of rat cortical homogenates had been successful, a more rapid and sensitive screen was possible with the cloned human H_3 receptor because of their higher density (typically >1 pmol/mg protein) compared to the rat (~100 fmol/mg protein). A number of new chemical series were identified, most of which have yet to be described. Most of these series displayed higher potency for the human receptor compared to the rat. This has led us to a counterintuitive focus in finding compounds with high rat affinity in these series in order to achieve balanced affinity. Cross-species equivalency of potencies is desirable to help rationalize that doses administered, exposure and receptor occupancy levels achieved in animal studies will be predictive for human clinical studies.

At the same time, the human receptor screens were being developed, we and others were learning much about the nature of compounds that bound with high affinity to H_3 receptors, and prototypic ligands generally consisted of an amine (typically still imidazole) linked (generally via a propyloxy group) to a lipophilic terminus. Ciproxifan is an excellent example of this core structure, and A-304121, despite its different origin, can be viewed as a piperazine analog of the imidazole, ciproxifan. Thus, we (and others) continued to explore this type of core molecule, and we synthesized a number of propyloxy-linked compounds, for example, A-331440 (4′-[3-(3(R)-(dimethylamino)-pyrrolidin-1-yl)-propoxy]-biphenyl-4-carbonitrile; Fig. 1; [21]) and A-349821 ([4′-{3-([R,R]2,5-dimethyl-pyrrolidin-1-yl)-propoxy}-biphenyl-4-yl]-morpholin-4-yl-methanone; Fig. 1; [22]). Both compounds were of high and relatively equal potency at both human and rat receptors and had novel amines taking the place of the traditional imidazole. Interestingly, the pharmacological profile of these compounds in animal studies was quite different, suggesting functional heterogeneity of H_3 receptors and of the roles they play. A-331440 had modest activity in an inhibitory avoidance model [23] that was developed to characterize H_3 receptor antagonists, a model that was dependent upon attentional and cognitive deficits found in spontaneously hypertensive rat pups, but not in other strains or age groups. A-349821 was very active in this model of cognitive enhancement [22]. Interestingly, A-331440 was highly efficacious in obesity models being developed within Abbott by colleagues in our Metabolic Diseases Research Group [24], whereas A-349821 has not demonstrated any weight loss effect, even in high dose toxicological studies (Hancock et al., unpublished observations). A hypothesis that we had to consider was of different subtypes of H_3 receptors, based on early work suggesting such a dichotomy from investigators at Schering-Plough [25]. Such considerations occurred at the same time that increasing levels of complexity were being appreciated for the H_3 receptor.

3. H_3 receptor splice isoforms

Comparison of the gene and protein sequences of H_3 receptors from various species indicated the presence of introns in several, but not all species (for review, see Ref. [26]). While the intricacies and variations of these splice isoforms are beyond the scope of this commentary, of the many isoforms identified in the various species studied few have been characterized pharmacologically. Some are presumed to be inactive in their own right, because they are truncated at portions of the receptor thought to be important for ligand recognition or receptor trafficking. However, some truncated H_3 receptors may have indirect effects to hinder the cell-surface expression of functional receptors [27]. More pharmacological details are available on human and rat isoforms in which portions of the third intracellular loop were deleted. This region of GPCRs is critical to association with and activation of G-proteins. While the H_3 receptor was originally thought to couple to inhibition of adenylate cyclase through G_i [4], studies have identified a number of additional signal-transduction pathways that are affected by H_3 receptors [7,26]. Thus, different isoforms (20 potential variants of the human H_3 [26]), which have different preferences for different signal-transduction pathways and apparently differential distributions in various brain regions [28,29], could respond differently to pharmacological agents. Our studies of two of these isoforms, hH_3(445) and hH_3(365) (for nomenclature, see Ref. [7]), have revealed only modest differences in affinities for a variety of compounds in radioligand binding assays [30], similar to findings of other groups (for reviews, see Refs. [7,26]). This is not surprising, since the ligand binding domain is probably minimally effected by the 80 amino acid deletion in intracellular loop 3. Thus, more detailed investigation through functional assays may be required to differentiate among isoforms.

Other complexities of the H_3 receptor include indications of polymorphisms (for review, see Refs. [7,26]). There is no known pharmacological consequence of H_3 receptor polymorphisms, although their presence should not be ignored, since other receptor polymorphisms have been linked to human disease, receptor processing, dimerization or desensitization, ligand binding and signal-transduction [31]. The impact of these phenomena on the pharmacology of H_3 receptor antagonists has barely been probed, to date.

4. H_3 receptor antagonists and compound-related adverse effects

With regard to our prototypic lead compounds, A-331440 and A-349821, additional challenges thwarted their advancement into clinical trials. For A-331440, favorable characteristics

included the balanced affinity at human and rat H_3 receptors, sustained efficacy in antiobesity tests, ready access to the CNS (generally in excess of blood levels by more than 100-fold) providing low circulating levels that were expected to reduce the risk of any adverse effects. However, A-331440 was genotoxic in an in vitro micronucleus test [32]. Fortunately, several analogs were found to pass the micronucleus test at high concentrations, and these compounds represent some of the best characterized of our antiobesity leads in both rat and mouse models [32]. In parallel, research investigators at Novo Nordisc have identified compounds with antiobesity effects in rodents and primates [33]. These findings would suggest that the basic pharmacology, relevant sites of action, neurotransmitter pathways, etc., are similar in both rodents and monkeys, a key finding since the monkey receptor may lack splice isoforms, has the human sequence at the key amino acids 119 and 122 [7], and may, therefore, be a useful predictor of human H_3 receptor pharmacology. It was also an important finding that compounds of a completely novel class (cinnamic amides), lacking the structural features of other known H_3 receptor antagonists, could have antiobesity effects in vivo.

The findings of antiobesity effects of structurally diverse H_3 antagonists also addressed key pharmacological concerns regarding target validation. Within the past decade, numerous investigators have used animals whose expression of a gene has been ablated to understand the role of that gene, based on phenotypic changes in the resultant knockout mice. For H_3 receptor knockout mice, various groups had reported quite distinct phenotypes with respect to body weight, ranging from lower [34] to higher body weights [35]. Our own limited studies had shown a modest increase in body weight [36] and a lack of effect of A-331440 on feeding in such animals. Obese H_3 receptor knockout mice might present a paradoxical phenotype, if mice lacking presynaptic H_3 histamine receptors would lose tonic inhibition of histamine synthesis and release resulting in over-stimulation of postsynaptic H_1 receptors and reduced food intake and body weight. However, thioperamide failed to reduce acute feeding responses in the H_3 receptor knockout animals, underscoring the importance of intact presynaptic H_3 receptors in order to demonstrate an antiobesity effect of H_3 receptor blockade [35], consistent with our own data on A-331440 in H_3 receptor knockout mice [36]. The lack of inhibition of food consumption is also an important finding, since one group has proposed that the effects of thioperamide on food consumption may be caused by taste-aversion type of disruption of normal feeding behavior [37]. However, it would be anticipated that taste aversive properties of compounds would be maintained in H_3 receptor knockout animals. Moreover, the Novo compound was reported to not disrupt feeding behavioral in a behavioral satiety sequence test [33]. These findings also underscore the challenges of understanding the role of receptors through knockout studies, where investigators have shown that the observed phenotype is not always as predicted, as found with other hypothalamic regulators of body weight [38].

In contrast to A-331440, A-349821, while active in our behavioral evaluation, also encountered an insurmountable obstacle in our pursuit of a suitable clinical candidate. A-349821 had very desirable attributes of low nanomolar affinity for H_3 receptors across all species tested, to date. The compound was extremely selective for H_3 receptors, was not genotoxic, had desirable pharmacokinetic properties, and was without overt toxicity in a 2-week rat toxicity test (Hancock et al., unpublished observations). In cardiovascular safety tests, A-349821 had an essentially benign cardiovascular profile at doses that were administered in vivo [Reinhart and Hancock, unpublished observations]. This was an encouraging finding when interpreted within the context of the findings of Levi and co-workers [39] who had shown beneficial effects of H_3 receptor agonists in models of myocardial ischemia. Unknown at the time were the potential effects of an H_3 receptor antagonist on cardiovascular function and cardiac performance. The lack of profound hemodynamic effects of A-349821 in the anesthetized dog model suggested that blockade of presynaptic H_3 receptors in the cardiovascular system would not lead to deleterious hemodynamic effects. However, in the canine cardiovascular safety evaluation, A-349821 did not have an adequate safety margin with respect to the QT interval, causing prolonged cardiac action potential duration despite relatively low binding affinity ($K_i > 1\ \mu M$) for human ERG potassium channels [Reinhart and Hancock, unpublished observations]. When A-349821 was administered in multiples of the blood levels associated with efficacy in cognition models, adverse electrophysiological effects were observed at only about 12× above efficacious blood levels. However, A-349821 also did not have ready access to the CNS, exhibiting blood and brain concentrations that were approximately equal [40], necessitating higher blood exposure levels for behavioral efficacy. Since the compound was not a *P*-glycoprotein substrate (Everitt and Hancock, unpublished observations), we subsequently reasoned that the amide nature of the molecule reduced CNS access. On the other hand, we found that A-349821, when tritiated, was an excellent radioligand, maintaining the high nanomolar affinities for H_3 receptors, irrespective of species, with low non-specific binding. Additionally, as a non-imidazole antagonist/inverse agonist radioligand [^{3}H]-A-349821 offers certain advantages over either traditional agonist radioligands, such as *N*-α-methylhistamine or imidazole-based antagonist ligands, such as iodophenpropit or iodoproxyfan [Witte and Hancock, unpublished observations]. To solve the cardiovascular safety issues observed with A-349821, we redirected our medicinal chemistry efforts again, seeking compounds with a better balance between CNS and peripheral levels, much as had been achieved with A-331440.

5. Enhancement of drug-like properties of H_3 receptor antagonists

Medicinal chemists often turn to one or more of several key concepts to increase the likelihood of identifying drug-like molecules. We had previously avoided imidazole-based compounds because of such reasoning, since many imidazoles are potent inhibitors of drug-metabolizing CYP enzymes, leading to the potential for drug–drug interactions, or inhibition of adrenal steroid production [41]. A recent pragmatic example demonstrates the importance of such drug–drug interactions. Augmentation of the cataleptogenic effect of haloperidol in the rat [42] may have resulted from inhibition of the metabolism of haloperidol, known to be

mediated by CYP3A4 [43], since potentiation of catalepsy was also observed with the imidazole H_3 antagonist thioperamide, but not with two non-imidazole-based H_3 antagonists [44]. Notably, the imidazoles displayed evidence of inhibition of the metabolism of haloperidol and risperidone, whereas non-imidazole compounds did not [44], supporting the importance of drug–drug interactions as a potential toxic side effect.

Based on our own findings and those of others [45] non-imidazoles are also much more selective for H_3 compared to H_4, and generally H_1 or H_2 receptors, since they lack the imidazole base of histamine. Moreover, some imidazoles have been reported to have poor CNS penetration. Other medicinal chemistry principles useful to enhance absorption, decrease metabolism, etc., include rigidification, or decreasing the number of rotatable bonds. This concept was applied to the aforementioned propyloxy-linking group, leading to a number of compounds with a benzofuran core [46]. Investigation of novel amines of compounds of this type led to a molecule with the amine substitutent, 2-*R*-methyl-pyrrolidine, namely ABT-239 (4-(2-[2-(2(*R*)-methyl-pyrrolidine-1-yl)ethyl]benzofuran-5-yl)-benzonitrile; Fig. 1). This compound is highly potent at H_3 receptors in all species tested to date (approximately 1 nM) and is highly selective for H_3 compared to all other receptors tested. However, in our initial examination of the binding affinity of this compound at native H_3 human receptors in brain cortical homogenates, ABT-239 exhibited approximately 10-fold lower affinity than expected at native human H_3 receptors based on its affinity in radioligand binding assays of the native rat cortical receptor or the cloned rat and human receptors. This lipophilic compound was highly protein bound, and there was considerably more protein present in the human cortical homogenates than in our other binding assays because of the low receptor levels in the human tissue homogenates. We hypothesized that ABT-239 might be non-specifically bound to CNS proteins and lipids. To examine that, ABT-239 was preincubated with human cortical homogenates, the homogenate mixture centrifuged and the binding affinity of the free ABT-239 in the supernatant was determined in a subsequent bioassay against cloned human H_3 receptors. The results suggested that as much as 90% of the compound was bound within the human tissue homogenate, and the affinity of ABT-239 at cloned human receptors, or at native receptors wherein the protein concentration was reduced, provided K_i values in the low nanomolar range.

Other drug-like features of ABT-239 included lack of genotoxicity, low index of phospholipidosis, desirable pharmacokinetic properties across all species tested, and safety in a 2-week rat toxicity assessment at high multiples over its exposure in behavioral assays. As was hoped, the compound had a much more favorable brain:blood ratio, generally in excess of 20-fold, but was cleared from the CNS within 24 h, essentially in parallel with blood concentrations [47]. This finding is often not the case with a variety of other H_3 antagonist compounds, particularly diamines that not only induce phospholipidosis, as mentioned above, but also have very long resident times within the CNS, as was recently reported for a diamine-based H_3 antagonist from Johnson and Johnson [48]. Other diamines [49], or unrelated compounds that

		Donepezil	Methylphen-idate	Risperidal	Thioper-amide	Ciprox-ifan	A-304121	ABT-239
Cognitive Domain ↓	Relevant Human Disease			--				
Memory Consolidation	**AD**	+		--	+	ND	ND	ND
Spatial Orientation	**AD**	+			+	+	ND	+
Working Memory	**AD**	+			+	ND	ND	ND
Attention Impulsivity	**AD** **ADHD**	+	+		+	+	+	+
Social Memory (Short-term Memory)	**ADHD**		+		+	+	+	+
Sensory Gating and Pre-attention	**Schizo-phrenia**			+	+	+	ND	+
Positive Symptoms	**Schizo-phrenia**			--	ND	+	ND	+

+ Indicates efficacy in behavioral models of these cognitive domains in

behavioral models for these specific compounds.

ND: Not Determined for these key well-characterized antagonists.

Fig. 2 – Broad efficacy in preclinical models of cognitive deficits with ABT-239. Animal models are used to analyze cognitive domains thought to be important for human diseases, such as Alzheimer's disease (AD), attention deficit hyperactivity disorder (ADHD), or schizophrenia [50]. Data for reference H_3 receptor antagonists are compared to the preclinical data for drugs used to treat AD (donepezil), ADHD (methylphenidate), or schizophrenia (risperidal). Modified from Ref. [50].

we synthesized in the course of our investigations (Kolasa et al., unpublished observations) may share the limiting problems of phospholipidosis and/or retention within the CNS.

An attractive feature of ABT-239 is that the compound demonstrated broad efficacy in multiple behavioral tests of cognitive deficits ([50]; Fig. 2), including those thought to be predictive of attention deficit hyperactivity disorder, Alzheimer's disease and schizophrenia. These behavior tests tap into multiple neurotransmitter systems and multiple cognitive domains [50], and predict that compounds like ABT-239 would have the potential for clinical efficacy in more than one human deficiency of cognition. The fact that ABT-239 was effective upon repeated administration (see below), did not cause CNS stimulation [40] and had a broad therapeutic index (Fig. 3) underscored the drug-like properties of this compound that are not necessarily observed in all H_3 receptor antagonists (Hancock et al., unpublished observations).

Unlike imidazoles, ABT-239 was not an inhibitor of any CYP enzymes studied [46], and was selective for the H_3 receptor, compared to hERG channels, by more than 100-fold. The latter findings predicted that the canine cardiovascular safety profile would be favorable. Indeed, no adverse cardiovascular effects were observed in the anesthetized dog model at concentrations of more than 180-fold above efficacious blood exposure levels [Reinhart and Hancock, unpublished observations]. Initial concerns that the compound appeared to be a substrate solely for CYP3A4 raised the specter of indirect drug–drug interactions, in that other compounds metabolized exclusively by CYP3A4 and/or inhibitors of the enzyme could interfere with the catabolism of ABT-239 and vice versa. However, in elegant experiments (Pan et al., unpublished observations), additional pathways were identified that contributed significantly to the metabolism of ABT-239, including flavin mono-oxygenases [46]. Thus, the likelihood of significant drug–drug interactions was dramatically reduced.

Subsequent cardiovascular safety studies in telemetered cynomolgous monkeys, however, were not as sanguine. Although no cardiovascular events were noted at doses of 0.1 and 0.33 mg/kg, p.o., oral doses of 1 mg/kg, expected to produce peak blood levels of about 158 ng/mL, were associated with QT prolongation in all animals [Reinhart and Hancock, unpublished observations]. Since the efficacious exposure levels in rat behavioral models ranged from 0.22 to 4.7 ng/mL [46], a conservative estimate of the margin of safety (158/4.7) was only 30-fold, which was deemed insufficient for clinical evaluation.

6. Further H_3 receptor antagonist considerations: repeated dosing efficacy and inverse agonism

While many selective antagonists for the H_3 receptor have now been described of quite diverse chemical structure, there are far less detailed biological data reported for most of these compounds. Moreover, their preclinical evaluation has

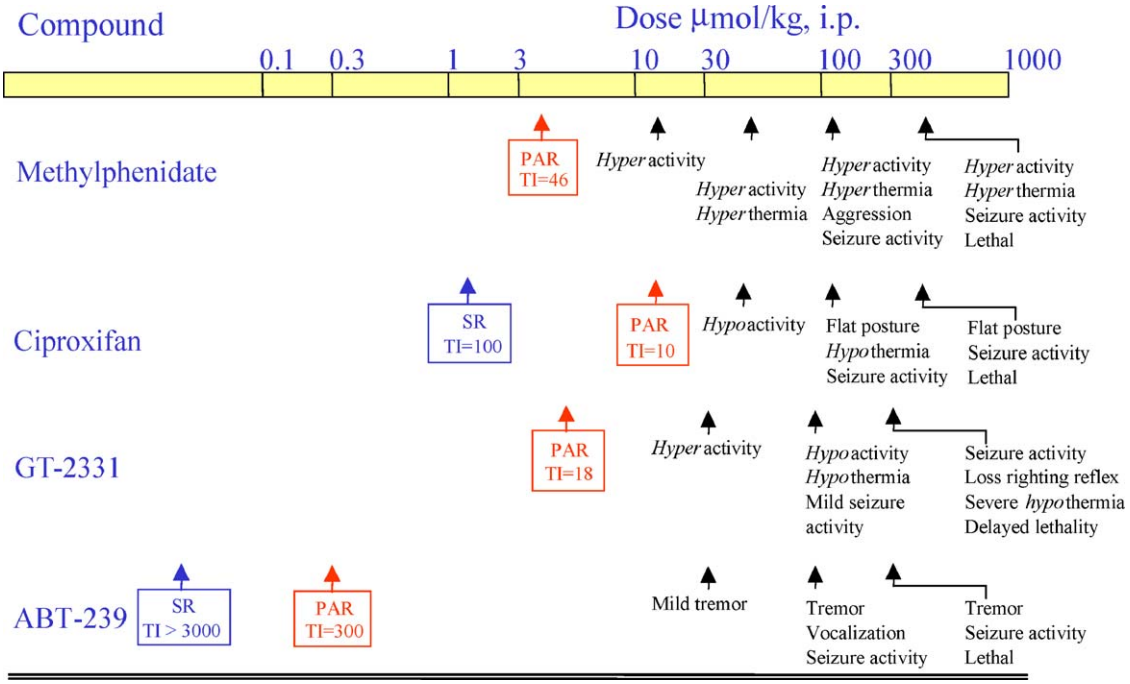

Fig. 3 – Improvement in safety margins among H_3 receptor antagonists. Ciproxifan, GT-2331, and ABT-239 are compared to methylphenidate, the reference agent for treatment of attention deficit hyperactivity disorder (ADHD). The five-trial passive avoidance response (PAR) model [23] was used as an animal model of the cognitive and attentional deficits of ADHD, whereas the social recognition model (SR [50]) has cognitive components that may be relevant to ADHD and other memory or learning deficits (see Fig. 2). For each compound, a preclinical therapeutic index (TI) was calculated, based on the ratio of the doses causing notable side effects (e.g., marked hyperactivity or tremor) to those active in the PAR or SR models. Compounds, such as A-304121, A-317920, A-331440, and A-349821 generated progressively greater TI values, equal to (A-304121), or greater than ciproxifan but less than ABT-239.

generally depended upon the interests of the research teams, i.e., models of sleep or cognitive performance or in feeding and weight loss, among others. Of course, this reflects the lack of clinical validation of the therapeutic utility of H_3 receptor blockade, in a sort of vicious circular Venn diagram. Within this context, we were concerned that the acute effects of H_3 antagonists might not be maintained upon repeated dosing. Such concerns arose because of early findings that ciproxifan, while showing an acute loss of body weight in rats, failed to maintain efficacy after 15 days dosing [51]. In the same study, A-304121 had no effect on body weight over the same time course. Did these data represent a dichotomy between antiobesity and cognition-enhancing compounds, or did it reflect an extremely rapid desensitization of H_3 receptors following administration of A-304121? While we had seen rapid desensitization of H_3 receptors following agonist administration in the guinea-pig ileum model [52,11], there was no obvious mechanism whereby antagonists would induce receptor desensitization. On the other hand, since H_3 receptor antagonists enhance the release of a variety of agonist neurotransmitters (including, of course, histamine itself), an indirect desensitization of one or more of downstream receptors could contribute to loss of effect upon repeated dosing of H_3 receptor antagonists. With respect to the antiobesity lead, A-331440, we were reassured to observe that repeated dosing of this compound, and several of its analogs, for periods of up to 28 days did not result in loss of efficacy [24,32]. In fact, these compounds show a cumulative effect on weight loss, predominantly of adipose tissue, upon repeated dosing, thus, representing an attractive profile for an antiobesity agent. With respect to cognitive enhancement, we have recently reported that ABT-239 was effective upon repeated dosing in an inhibitory avoidance model using spontaneously hypertensive rat pups as a model for attention deficit hyperactivity disorder-related cognitive deficits [40]. The extent to which this is the case for other cognition-enhancing H_3 receptor antagonists is unknown, as is any mechanistic understanding of how such compounds would differentiate from similar compounds that maintain efficacy upon repeated dosing, and requires continued vigilance and additional mechanism of action studies.

A key molecular analysis of the H_3 receptor peptide sequence [53] revealed a 12-amino acid region analogous to that of receptors mutated to become constitutively active (for review, see Ref. [7]). The findings of constitutive activity of the native receptor established a framework for further analysis of pharmacological responses. On the one hand, constitutively active receptors raised the likelihood that compounds, hitherto thought to be antagonists, could manifest activity as inverse agonists. Indeed, this has been observed for a number of compounds of various classes. Moreover, some compounds could be expected to act as protean agonists [54], where the nature of their pharmacological effect would depend on the assay context. Protean effects could rationalize why we and others [55], have observed compounds, such as GT-2331 (4-[2-(5,5-dimethyl-hex-1-ynyl)-(1*S*,2*S*)cyclopropyl]-1H-imidazole; Fig. 1) to have antagonist, agonist, partial agonist, or inverse agonist effects in various test modes. Moreover, we have observed that the degree of constitutive activity differs between species (rat versus human) and between the different splice isoforms, such that ''antagonists'' may demonstrate differential degrees of inverse agonist activity in different in vitro assays [56,57].

A second important consequence of inverse agonism at H_3 receptors relates to how these receptors may function in different brain regions. The concept of constitutive activity predicts that the H_3 receptor will be active in the absence of competing ligands. Thus, in histamine-poor areas of the brain, the receptor would function independently of competing histamine. This should lead to H_3-receptor-mediated inhibition of the release of many neurotransmitters, e.g., acetylcholine, dopamine, serotonin, or norepinephrine, among others. Activation of H_3 heteroreceptors should not be influenced by the synaptic concentrations of these neurotransmitters, since they would be expected to have low affinity for H_3 receptors (although this could be less true for acetylcholine, perhaps, based on the original classification of the H_3 as the muscarinic M_6 receptor by Millennium (vide supra). Likewise, the effect of inverse agonists, to block the H_3 heteroreceptor to enhance the release of these diverse neurotransmitters, should be unaffected by their increased synaptic concentrations. In contrast, in areas of the brain that are enriched in histamine (such as the hypothalamus), synaptic histamine release should inhibit the presynaptic synthesis and release of histamine [2]. Exposure to an inverse agonist should enhance the release of histamine into the synapse, which then may be available to compete with the inverse agonist for occupancy of the presynaptic autoreceptors and/or lead to desensitization of these receptors. It is not difficult to envision a very complex, shallow dose–response relationship in which the effect of an inverse agonist on histaminergic terminals would increase synaptic histamine to reduce the efficacy and apparent potency of the inverse agonist to a greater extent than would be the case at an H_3 heteroreceptor where no such competition would occur. Since H_3 receptors are distributed so widely throughout the CNS and in regions wherein histamine levels (and perhaps free concentrations) are different [58–60], may have circadian changes [59], or change with pathophysiology [61], the efficacy of a given concentration of an H_3 antagonist/inverse agonist at any H_3 auto- or heteroreceptor may be quite different and may depend on a variety of factors. Moreover, the distribution of H_3 antagonists within various brain regions is not necessarily homogeneous (Milicic and Hancock, unpublished observations), adding a further layer of complexity to these interactions.

7. Conclusions

The aforementioned findings of species-related sequence differences, pharmacological differences, splice isoforms, constitutive activity, desensitization mechanisms, hetero-, and autoreceptor effects all add to the routine concerns in drug development of safety issues, drug metabolism issues, and pharmacological activity that seem particularly byzantine in the case of the histamine H_3 receptor. Given these complexities, it is less surprising, perhaps, that no compound in this class has provided that essential clinical proof-of-concept. Our own challenges with genotoxicity, phospholipidosis, cardiovascular safety, or metabolic interactions, even

with compounds that were optimized to reduce these risks, are indicative of the challenges of drug discovery with novel GPCR targets. Those of us working with these complexities may take small comfort from the observation that species typical of the drug discovery process express the H_3 receptor, unlike the challenges faced by investigators of the recently de-orphanized and related human receptors GPR7 and GPR8, wherein there appears to be no rodent homolog to GPR8 [62]. This absence could make interpretation of the role of receptor ligands and their selectivity a challenge for drug development. For the H_3 receptor, we are continuing to seek compounds with reduced risks and improved likelihood for successful advancement through the rigors of clinical trial. As such, compounds need to maintain the excellent preclinical profile of examples like ABT-239, lacking overt toxicity, genotoxicity, phospholipidosis potential metabolic liabilities, with benign cardiovascular profiles, excellent pharmacokinetic and pharmacodynamic properties, with favorable brain:blood ratios, etc. Clearly, an area to improve upon would be the selectivity for the H_3 receptor compared to ion channels involved in controlling cardiac action potential. In this regard, we have discovered that chemical modifications [Cowart and Hancock, unpublished observations] to the core structure of compounds like ABT-239 can dramatically increase their selectivity for the H_3 receptor compared to the hERG channel while maintaining or improving upon their other drug-like characteristics. We have also discovered a number of replacement cores to the benzofuran moiety of ABT-239 that maintain these same favorable attributes and/or enhance CNS access, with the goal of reducing further the peripheral exposure levels associated with efficacious CNS levels. Examples of benzofuran replacements include naphthyls [63], or heterocyclic ring systems [64] that lead to drug-like molecules. These compounds are among the most potent and selective H_3 antagonists described to date. We, like others, impatiently wait for signals of clinical success or failure with one or more novel H_3 antagonists now being advanced by various pharmaceutical companies.

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