Chemical Research in Toxicology

Article

pubs.acs.org/crt

Carbamate Insecticides Target Human Melatonin Receptors

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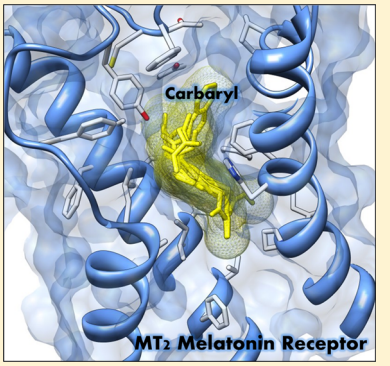
Supporting Information

ABSTRACT: Carbaryl (1-naphthyl methylcarbamate) and carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate) are among the most toxic insecticides, implicated in a variety of diseases including diabetes and cancer among others. Using an integrated pharmacoinformatics based screening approach, we have identified these insecticides to be structural mimics of the neurohormone melatonin and were able to bind to the putative melatonin binding sites in MT_1 and MT_2 melatonin receptors *in silico*. Carbaryl and carbofuran then were tested for competition with 2-[^{125}I]-iodomelatonin (300 pM) binding to hMT_1 or hMT_2 receptors stably expressed in CHO cells. Carbaryl and carbofuran showed higher affinity for competition with 2-[^{125}I]-iodomelatonin binding to the hMT_2 compared to the hMT_1 melatonin receptor (33 and 35-fold difference, respectively) as predicted by the molecular modeling. In the presence of GTP (100 μM), which decouples the G-protein linked receptors to modulate signaling, the apparent efficacy of carbaryl and carbofuran for 2-[^{125}I]-iodomelatonin binding for the hMT_1 melatonin receptor was not affected but significantly decreased for the hMT_2 melatonin receptor compatible with receptor antagonist/inverse agonist and agonist efficacy, respectively. Altogether, our data points to a potentially new mechanism through which carbamate insecticides carbaryl and carbofuran could impact human health by altering the homeostatic balance of key regulatory processes by directly binding to melatonin receptors.



INTRODUCTION

The nightly release of melatonin from the pineal gland is regulated by biological clocks in the suprachiasmatic nucleus (SCN) of the hypothalamus and transmits signals to the brain and peripheral target tissues through activation of the G-protein coupled melatonin receptors MT_1 and/or MT_2.[1−3] Rhythmicity of pineal melatonin synthesis from serotonin is tightly orchestrated by *N*-acetyltransferase (AA-NAT) and hydroxyindole-*o*-methyltransferase (HIOMT) both of which have peak enzymatic activities during the night.[4−6] Activation of melatonin receptors play a pivotal role in modulating the phase and amplitude of circadian rhythms phase and amplitude through the body.[1,7,8] Circadian rhythm misalignment is implicated in various conditions such as jet lag, delayed sleep phase syndrome (DSPS), advanced sleep phase syndrome (ASPS), and seasonal affective disorders (SAD).[9] Activation of melatonin receptors inhibit cyclic adenosine monophosphate (cAMP) formation,[10−13] regulate phosphorylation of the cAMP responsive element-binding protein (CREB),[14] protein kinase activities,[15,16] and ion fluxes.[12,17] In essence, the melatonin receptor system regulates a wide-array of cellular signaling events essential to maintain rhythmicity and homeostatic balance of various regulatory processes.[3,18] Aberrant melatonin receptor activation, sensitivity, and trafficking are linked to pathological conditions including cancer,[19] diabetes,[2,20] and cardiovascular disease.[21]

Exposure to chemicals that are structurally similar to melatonin can significantly alter this balance and could result in alteration of melatonin mediated signaling[3] and potentially other conditions that impact human health. In the present study, innovative combinations of computational tools for chemical clustering of large data sets of environmental chemicals were employed in tandem with predictive three-dimensional models in an attempt to identify new-class of environmental neuroendocrine disruptors targeting melatonin receptors. Briefly, environmental chemicals that could potentially interact with melatonin receptors based on their structural similarity were identified using an integrated pharmacoinformatics screen from a comprehensive Chem2Risk knowledgebase. Molecular docking of select environmental modulators established novel bimolecular interactions of carbamate insecticides carbaryl and carbofuran with the MT_1 and MT_2 melatonin receptors *in silico*. Furthermore, the current study investigated ligand affinity, selectivity, and apparent intrinsic efficacy of selected environmental disruptors on hMT_1 and hMT_2 melatonin receptors *in vitro*.

Agricultural use of carbamates as insecticides is due to their strong yet reversible inhibitory effect on acetylcholinesterase.[22] Occupational exposure to carbamates has been reported to be associated with psychological distress and depression among agricultural workers.[23,24] There is also a positive correlation between carbamate exposure and the risk of developing diabetes and metabolic syndrome.[25] Carbaryl produces behavioral changes in rodents such as tremor[26] and hypothermia.[27] Furthermore, a recent study reports age-specific

Received: August 30, 2016
Published: December 27, 2016

DOI: 10.1021/acs.chemrestox.6b00301
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sensitivity to carbaryl in Brown-Norway rats indicative of age-related changes in metabolism. Even low-dose (3 mg/kg) carbaryl treatment exhibits these age-specific differences in both biochemical and behavioral experiments.[28] While some of these effects were attributed to cholinesterase inhibition and neurotoxicity at higher doses, persistent exposure to lower doses of insecticides including carbamates in both occupational and nonoccupational environment was associated with increased risk of various disease conditions including diabetes, cancer, and depression.[24,25] Center for Disease Control (CDC) reports that in addition to the dermal routes (the most common route of exposure), several classes of insecticides including the carbamates that are used in spray formulations can reach the brain and other target tissues via inhalation, circumventing liver metabolism (CDC ToxGuide, 2003) and repeated exposure to these chemicals (as in occupational exposure) tend to render higher concentrations of insecticides in target tissues. It is imperative to find alternative targets and mechanisms through which carbamates and other insecticides render such a wide range of biochemical and behavioral effects.

MATERIALS AND METHODS

Pharmacoinformatics. Chem2Risk, a curated pharmacoinformatics knowledgebase of about 130,000 environmental chemicals with established 2D/3D structure coordinates was generated from US Environmental Protection Agency's (EPA) aggregated computational toxicology resource (ACToR ~500,000 chemicals), ChEMBL (1 million chemicals),[29] STITCH3.0 (~300,000 chemicals),[30] and CTD (12,000 chemicals)[31] along with an in house knowledgebase of environmental agents (~170,000 chemicals). The ACToR database contains ToxREf, ToxCast DSSTox, and EpoCast databases and compounds from over 1000 public sources and represents several years of *in vitro* and *in vivo* toxicity screening studies.

Chemical Similarity Clustering. Chemical similarities were computed using the Tanimoto coefficient, Dice's coefficient, and Tversky index.[32] Chemical clustering was performed using ChemMineR tools.[33] The distance matrices were calculated by all-against-all comparisons of chemicals using atom pair similarity measures and transforming the generated similarity scores into distance values with respect to the seed molecule (melatonin).

Molecular Docking of Environmental Melatonin Ligands. The strength of the chemical clustering was bolstered by *in silico* screening of potential *environmental* melatonin ligands against predictive theoretical models of melatonin receptors. Melatonin receptors hMT_1 (350 residues) and hMT_2 (362 residues) are seven transmembrane proteins connected via several intra- and extracellular loops, with approximately 60% overall sequence homology and 73% sequence homology in their transmembrane domains.[3] None of the crystallized GPCR structures shares more than 30% sequence identity with melatonin receptors. The human β_2 adrenergic receptor ($h\beta_2AR$) has 34% and 32% sequence similarity with hMT_1 and hMT_2 melatonin receptors, respectively. The high resolution X-ray crystal structure of $h\beta_2AR$ (PDB ID: 2RH1) was chosen as the template as it provides 82% (residue range 23−298) and 78% (residue range 36−324) sequence-structure coverage with MT_1 and MT_2, respectively. Several theoretical models of MT_1 and MT_2 were generated using Modeller9v8.[34] The putative binding pockets for MT_1 (inclusive of residues H205, S110, and S112) and for MT_2 (inclusive of residues N175, H208, N268, and Y298) were inferred from mutagenesis data.[3] Melatonin receptor models were superimposed with the template, and initial orientations of putative binding site residues were visually inspected (Figure S1A). TM3 and TM5 of MT_1 and MT_2 models were refined based on a knowledge-based helical search as described by Akula and Pattabiraman.[35] Stereochemical quality of the generated models was assessed using the PROCHECK application. PDB structures were prepared for molecular docking experiments using the protein structure preparation tool followed by Protonate3D as implemented in the Molecular Operating Environment (MOE, Chemical Computing Group Inc., Montreal, Canada). These structures were subjected to a short energy minimization routine to relax the structures using Amber 99 force field as implemented in MOE. Surflex-Dock (SYBYL, Cerata USA, Inc., Princeton, NJ), VINA (Autodock, Molecular Docking, The Scripps Research Institute), and MOE docking suite were employed to dock select environmental chemicals against human melatonin receptor models. A mini-library of 25 prototype melatonergic ligands with reported experimental binding affinities (ranging from 0.013 to 307.35 nM) retrieved from G -Protein Ligand Database (GLIDA)[36] were docked into MT_1 and MT_2 receptor models. Melatonin receptor models with maximum number of ligand poses scoring similar or better than the cognate ligand melatonin and docked accurately into the putative melatonin binding pocket were chosen for further molecular docking experiments. Molecular visualization and graphics program Chimera was used to generate images.[37]

Materials. cDNA containing the complete coding region of the hMT_1 (human Mel_{1a} cloned into pcDNAI) and hMT_2 melatonin receptor (human Mel_{1b} cloned into pcDNA-3) were provided by Drs. S. M. Reppert and D. Weaver (Department of Neurobiology, University of Massachusetts Medical School, Worcester, MA, USA).[10,38] Effectene transfection kit was obtained from Qiagen (Valencia, CA, USA). Cell culture products were obtained from Life Technologies (Carlsbad, CA, USA) and Corning (Manassas, VA, USA). 2-[^{125}I]-Iodomelatonin (SA: 2200 Ci (81.4TBq)/mmol) was purchased from PerkinElmer (Waltham, MA, USA). Carbaryl and carbofuran were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Carbaryl (13 mM stock solution) was dissolved in ethanol, and carbofuran (13 mM stock solution) was dissolved in dimethyl sulfoxide (DMSO). Carbaryl and carbofuran were next diluted to 1.3 mM in 50% ethanol/50% Tris-HCl buffer (50 mM and 10 mM $MgCl_2$ at pH 7.4 and 25 °C). Further dilutions were performed in Tris-HCl buffer. The ethanol or DMSO concentration in assays with 1 mM, 0.1 mM, or 0.01 mM carbamate compounds was 8%, 4%, or 0.4% respectively, producing minimal inhibition of 2-[^{125}I]-iodomelatonin binding when tested alone.

FLAG Tagging and Stable Transfection. The development of CHO-hMT_1-FLAG and CHO-hMT_2-FLAG stably expressing the human MT_1 and MT_2 melatonin receptor on the cell membrane was described previously.[39] Expression of melatonin receptors on the cell membrane is nicely visualized on cells transfected with either hMT_1-GFP or hMT_2-GFP (GFP: green fluorescent protein) showing significant membrane expression (see Figure 6 in ref 39).

Cell Culture. CHO cell cultures were grown as monolayers in Hem's F12 media supplemented with 10% fetal calf serum, 1% penicillin (10,000 IU/mL)/streptomycin (10,000 μg/mL) in 5% CO_2 at 37 °C. CHO-MT_1 and CHO-MT_2 cells were grown for 4 days until 90−95% confluence was reached. Cells were washed with PBS on ice, lifted in potassium phosphate buffer (10 mM, pH 7.4) containing 0.25 M sucrose and 1 mM EDTA, and then pelleted by centrifugation (4000 rpm, 5 min). Pellets were stored at −80 °C until used.

2-[^{125}I]-Iodomelatonin Binding Studies. Cell pellets were resuspended in 50 mM Tris-HCl (pH 7.4; 10 mM $MgCl_2$), homogenized, pelleted, and washed by centrifugation (20,000−30,000g). 2-[^{125}I]-Iodomelatonin binding was determined in cell membranes (5−10 μg protein/assay) as previously described.[11] Binding reactions were started by adding cell membranes to tubes containing binding buffer (50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$), 2-[^{125}I]-iodomelatonin (SA:2200 Ci/mmol, PerkinElmer, Waltham, MA, USA) (300 pM) and appropriate concentrations of vehicle (20 μL) or competing agents (20 μL) in a total assay volume of 0.26 mL. Following incubations for 1 h at 25 °C, reactions were terminated by rapid vacuum filtration through glass microfiber filters (Whatman, Krackeler Scientific, Inc., Albany NY, USA) soaked in 0.5% polyethylenimine solution. Filters were washed twice with 5 mL of ice-cold 50 mM Tris-HCl, dried, and the radioactivity determined on a gamma counter.

GTP Shift Assay. Membrane pellets were resuspended in 50 mM Tris-HCl and 3 mM $MgCl_2$, pH 7.4, to yield approximately 6−16 μg of

DOI: 10.1021/acs.chemrestox.6b00301
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protein/assay. 2-[^{125}I]-Iodomelatonin (SA:2200 Ci/mmol, PerkinElmer, Waltham, MA, USA) binding was carried out as described above in Tris buffer (50 mM Tris-HCl and 10 mM $MgCl_2$, pH 7.4), either in the absence or presence of 100 μM GTP, 1 mM EDTA, 150 mM NaCl, and appropriate concentrations of vehicle or competing agents [carbaryl (100 pM−1 mM) and carbofuran (100 pM − 1 mM)]. After 1 h of incubation at 25 °C, the reaction was terminated by rapid vacuum filtration and radioactivity measured as described above.

Data and Statistical Analysis. Competition curves were generated by nonlinear regression analysis using GraphPad Prism version 6 for Windows (GraphPad Software, La Jolla CA, USA). Results were expressed as $K_i = IC_{50}/(1 + [L]/K_D)$ where [L] is the concentration of the radioligand used in the assay and K_D the dissociation constant of the radioligand characterizing the membrane preparation. The apparent affinity shifts induced by GTP were measured by the ratio of K_i values ($K_{iGTP}/K_{icontrol}$) and classified as agonists (ratio >1), antagonists (ratio = 1), or inverse agonists (ratio <1).[40,41]

RESULTS

Chemical Clustering of Environmental Toxins. Structural similarity analysis of the Chem2Risk knowledgebase of suspect environmental chemicals using a modified ChemmineR interface[33] resulted in 71 compounds clustered with melatonin including hydroxychalcones, flavones, tamoxifen, carbaryl, and carbofuran (highlighted in gray, Figure 1). A majority of the

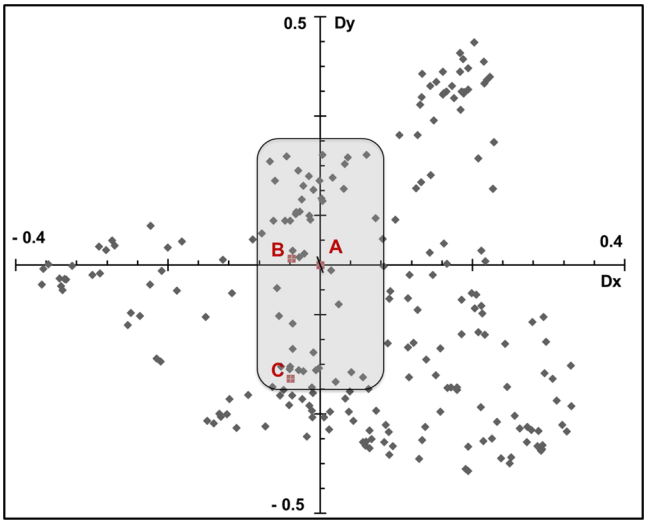

Figure 1. Chemical clustering of environmental chemicals from Chem2Risk Knowledgebase. Structural distances from the seed ligand melatonin (Dx, Dy) were computed using ChemMine tools. Melatonin, carbaryl, and carbofuran are marked A, B, and C, respectively.

coclustered environmental chemicals were from EPA's DSSTox database and had at least one report classifying them as a potential nuclear receptor ligand. Carbamates (Figure 2) were chosen for further analyses as they had weak to no reported nuclear receptor activity[42] and the highest pharmacophoric similarity to melatonin (Figure 2). Both carbamate insecticides share a hydrophobic moiety, a hydrogen bond acceptor, a hydrogen bond donor, and an aromatic moiety (Figure 2D−F).

Molecular Docking of Carbamates to hMT_1 and hMT_2 Melatonin Receptor Binding Sites. Melatonin and the carbamates were scored after docking into the putative melatonin binding pockets in hMT_1 and hMT_2 receptor models. Melatonin docks into the putative melatonin binding site with H195 (in hMT_1) or H208 (in hMT_2) within 2−3 Å (Figure S1B−C). Carbaryl consistently ranked higher than carbofuran but lower than melatonin in all of the scoring functions employing affinity dG (MOE) and surflex-dock scoring functions (SYBYL). Surflex docking experiments yielded at least five docked poses per receptor−ligand complex where the ligand was correctly positioned into the putative binding pocket and was chosen for further analysis. Molecular docking scores of melatonin−melatonin receptor complexes were significantly higher than that of the carbamates. Surflex docking scores of the five top ligand-hMT_2 receptor complexes were melatonin (11 ± 0.6) > carbaryl (8.7 ± 0.5) > carbofuran (7.9 ± 0.2), whereas the corresponding scores for the ligand-hMT_1 complexes were considerably lower for the insecticides [melatonin (11 ± 0.9) > carbaryl (6.3 ± 0.7) > carbofuran (4.7 ± 0.4)]. Autodock binding affinities (kcal/mol) of top insecticide-MT_2 complexes were −7.7 (carbaryl) and −5.8 (carbofuran) compared to −11.5 for the melatonin-MT_2 complex consistent with the results of surflex docking scores.

Competition of Carbamates for 2-[^{125}I]-Iodomelatonin Binding to Human Melatonin Receptors. The affinities of both carbaryl and carbofuran for competition to 2-[^{125}I]-iodomelatonin binding were determined for both hMT_1 and hMT_2 melatonin receptors stably expressed in CHO cells. Carbaryl (10 nM−1 mM) and carbofuran (10 nM−10 mM) competed with 2-[^{125}I]-iodomelatonin binding (300 pM) to membranes from CHO cells stably expressing either human recombinant hMT_1 or hMT_2 melatonin receptors[40] (Figure 3C and F). Table 1 shows affinity constants (K_i values) for the competition of melatonin, carbaryl, and carbofuran for 2-[^{125}I]-iodomelatonin binding to the hMT_1 and hMT_2 melatonin receptors. The affinity of carbaryl for the hMT_2 receptor was 33-fold higher than the hMT_1 melatonin receptor, while carbofuran showed a 35-fold difference in binding affinity between the hMT_1 and hMT_2 receptors (Table 1; Figure 3C,F). Both pesticides showed selectivity for hMT_2 over hMT_1; however, carbaryl showed higher affinity for either the hMT_1 or the hMT_2 receptors when compared with that for carbofuran (Table 1). We should note that the affinity of melatonin competition for 2-[^{125}I]-iodomelatonin was 8,100 and 233 times higher than that for carbaryl on the hMT_1 and hMT_2 melatonin receptors, respectively (Table 1).

Competition of Carbaryl and Carbofuran for 2-[^{125}I]-Iodomelatonin Binding to CHO-hMT_1 and CHO-hMT_2 Cells in the Presence of GTP. GTP shift assays are useful to determine the apparent "intrinsic activity" of ligands by the shifts in apparent affinity in the presence of GTP, which is known to decouple the receptor from the G-protein and in turn promote the stimulation or inhibition of signaling. The shifts in apparent binding affinities of carbamates were determined in the presence of GTP (100 μM) during competition with 2-[^{125}I]-iodomelatonin binding to hMT_1 and hMT_2 melatonin receptors (*affinity decreases* for agonists, *no change* for antagonists, and *increases* for inverse agonists).[41] GTP did not modify the apparent binding affinity of carbaryl for hMT_1, while it significantly decreased it for the hMT_2 (Figure 4A,B and Table 2). The affinity ratio GTP/control for carbaryl $K_{iGTP}/K_{iControl}$ was 0.65 ± 0.16 (n = 5) for the hMT_1 (Figure 4A and Table 2), while the ratio $K_{iGTP}/K_{iControl}$ for that of hMT_2 was 4.82 ± 0.82, which is consistent with ratios expected for ligands with an antagonist/inverse agonist or agonist (Figure 4B and Table 2). The apparent affinity of carbofuran for the hMT_1 melatonin receptor was not affected in the presence of GTP,

DOI: 10.1021/acs.chemrestox.6b00301
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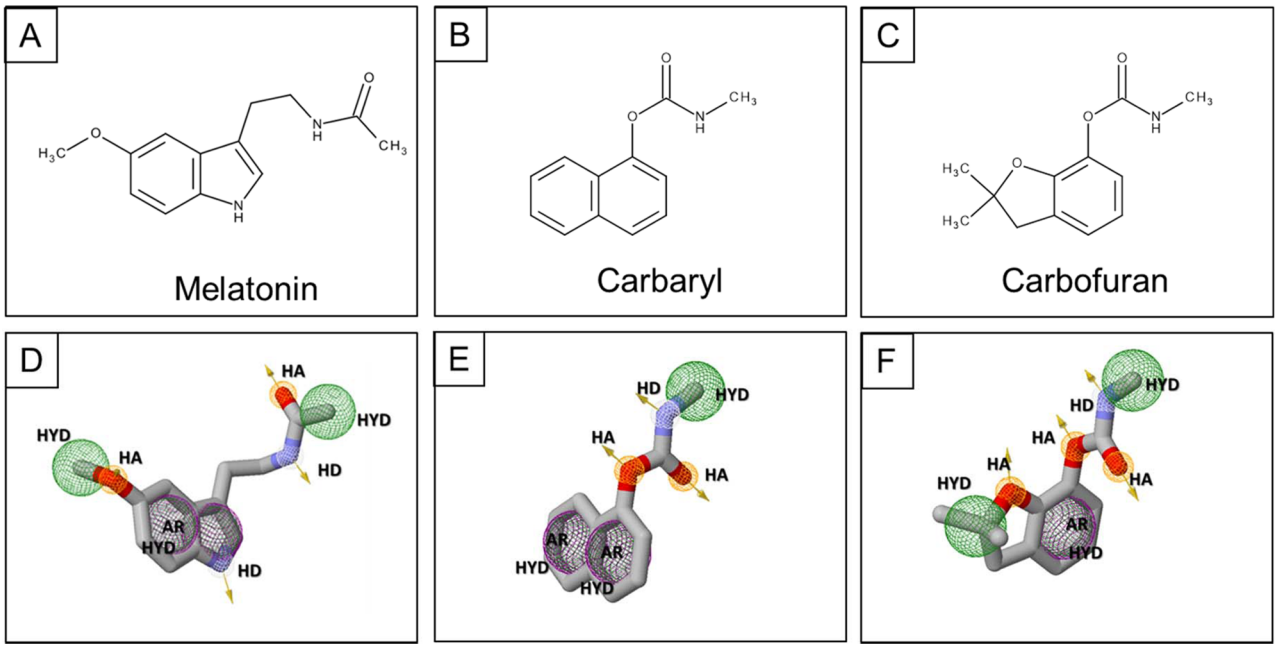

Figure 2. Chemical structures of (A) melatonin, (B) carbaryl, and (C) carbofuran. Three dimensional pharmacophoric similarities of carbamate insecticides (E) melatonin, (D) carbaryl, and (E) carbofuran. HA = hydrogen bond acceptor; HD = hydrogen bond donor; AR = aromatic moiety; and HYD = hydrophobic moiety.

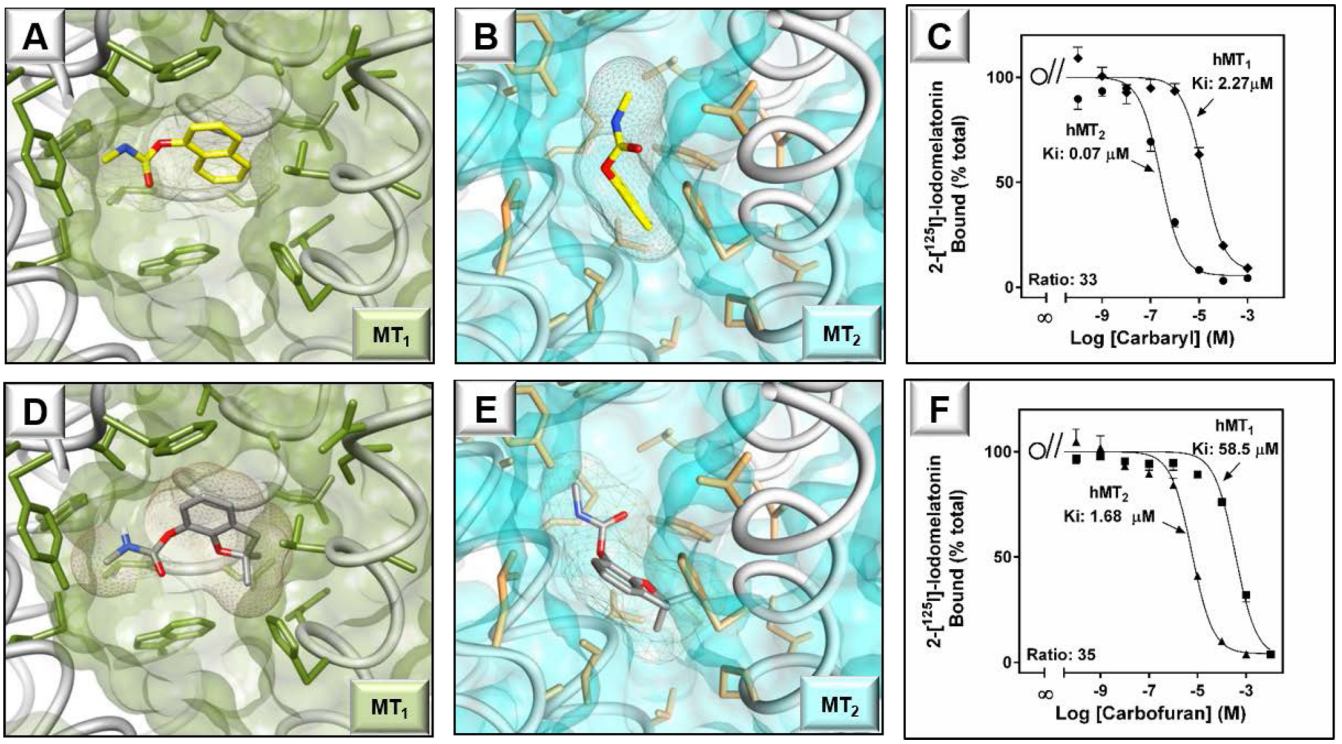

Figure 3. Carbaryl and carbofuran docking to and competition for 2-[^{125}I]-iodomelatonin binding to the hMT$_1$ and hMT$_2$ melatonin receptors. Binding poses of carbaryl-hMT$_1$ (A) and hMT$_2$ (B) or carbofuran-hMT$_1$ (D) and hMT$_2$ complexes (E). The ordinate represents 2-[^{125}I]-iodomelatonin binding to either the hMT$_1$ or hMT$_2$ receptors expressed as percent of total binding (C). Membranes from CHO cells stably expressing hMT$_1$ and hMT$_2$ receptors were incubated with 2-[^{125}I]-iodomelatonin (300 pM) in the absence (open circle) and presence (closed diamond for MT$_1$; closed circle for MT$_2$) of different concentrations of carbaryl (0.1 nM to 1 mM). The ordinate represents 2-[^{125}I]-iodomelatonin binding to either the hMT$_1$ or hMT$_2$ melatonin receptors expressed as percent of total binding (F). Membranes from CHO cells stably expressing hMT$_1$ and hMT$_2$ melatonin receptors were incubated with 2-[^{125}I]-iodomelatonin (300 pM) in the absence (open circle) and presence (closed square for MT$_1$; closed triangle for MT$_2$) of different concentrations of carbofuran (0.1 nM to 1 mM). The effect of vehicle (ethanol or DMSO) used to dissolve the carbamate compounds in competition for 2-[^{125}I]-iodomelatonin binding to the hMT$_1$ and hMT$_2$ was negligible and hence was not plotted. Values are the mean ± SEM of 8 independent experiments.

DOI: 10.1021/acs.chemrestox.6b00301
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Table 1. Affinity Constants for Melatonin, Carbaryl, and Carbofuran Insecticides for Competition with 2-[^{125}I]-Iodomelatonin Binding to hMT$_1$ and hMT$_2$ Melatonin Receptors Expressed in CHO Cells

	2-[^{125}I]-iodomelatonin binding		
	hMT$_1$[a]	hMT$_2$[b]	ratio[c]
melatonin receptor ligands	K_i value (μM)	K_i value (μM)	MT$_1$/MT$_2$
melatonin	$(0.38 \pm 0.016) \times 10^{-3}$	$(0.3 \pm 0.01) \times 10^{-3}$	0.86
carbaryl	2.27 ± 0.33	0.07 ± 0.01	32.4
carbofuran	58.5 ± 8.25	1.68 ± 0.24	34.8

[a]Competition of melatonin, carbaryl, or carbofuran for 2-[^{125}I]-iodomelatonin binding to CHO cell membranes stably expressing the hMT$_1$ and hMT$_2$ melatonin receptors. Binding was performed at 25 °C for 1 h. [b]K_i values were calculated from IC_{50} values obtained from competition curves using Cheng-Prusoff's equation.[64] K_i values are expressed as the mean ± SEM of 5−8 independent experiments (n = 5 for melatonin (MT$_1$), n = 6 for melatonin (MT$_2$), n = 8 for both carbaryl and carbofuran). [c]Ratio MT$_1$/MT$_2$ shows fold difference in affinity (K_{iMT1}/K_{iMT2}) of carbaryl and carbofuran for 2-[^{125}I]-iodomelatonin binding to the human MT$_1$ and MT$_2$ melatonin receptor.

showing a ratio [$K_{iGTP}/K_{iControl}$ = 0.59 ± 0.24] (Figure 4C, Table 2) compatible with those expected for an antagonist/inverse agonist. However, GTP significantly decreased the apparent affinity of carbofuran for the hMT$_2$ receptor suggesting agonist efficacy ($K_{iGTP}/K_{iControl}$ = 10.8 ± 4.2) (Figure 4D and Table 2).

DISCUSSION

Chemical clustering of environmental toxins in the Chem2Risk knowledgebase led to the identification of two carbamate insecticides with the highest pharmacophoric similarity to melatonin. These two carbamates, carbaryl and carbofuran, docked into the putative melatonin binding pockets in hMT$_1$ and hMT$_2$ receptor models with higher affinity for the hMT$_2$ receptors, which was verified in competition studies of 2-[^{125}I]-iodomelatonin binding to the human melatonin receptors. Lastly, both carbamates show distinct apparent intrinsic efficacy to the hMT$_1$ and MT$_2$ melatonin receptors. Together, these results suggest that unexpected exposure to these environmental chemicals may disrupt the homeostatic balance between neurotransmitter and modulators in target tissues expressing melatonin receptors.

Carbamate Insecticides Are Structural Mimics of Melatonin. Carbaryl and carbofuran coclustered within the same zone with melatonin (Figure 1) indicating high structural similarity. The pharmacophoric similarities of both carbamates with melatonin were also strikingly similar, indicative of potential molecular mimicry. Relative *in silico* binding affinities of the top hMT$_1$ or hMT$_2$ bound carbamate insecticides were 20−30% lower than that of melatonin. While the cognate ligand melatonin shows no preference toward hMT$_1$ or hMT$_2$, both carbamate ligands showed greater preference to hMT$_2$ receptor over hMT$_1$. The enhanced binding of the carbamate pesticides with hMT$_2$ can be attributed to the ring stacking interactions with H208 and partial occupancy of the hydrophobic cavity formed by the residues V124, I125, P212, I213, and F260 (Figure 3A,B,D,E). These ligand−receptor interactions were similar to the binding modes and interactions described for the selective MT$_2$ antagonist UCM454 and the selective partial agonist acylaminoethyl tetralin's interactions with hMT$_2$ receptors.[43,44] The carbamate insecticides carbaryl and carbofuran, docked very similarly to the putative binding modes suggested for prototype melatonergic ligands with hMT$_2$ receptors[45] clearly demonstrating the value of integrated clustering for rapid compound filtering and identification of environmental ligands that share chemical and pharmacophoric similarity prior to molecular docking experiments from very large compound databases.

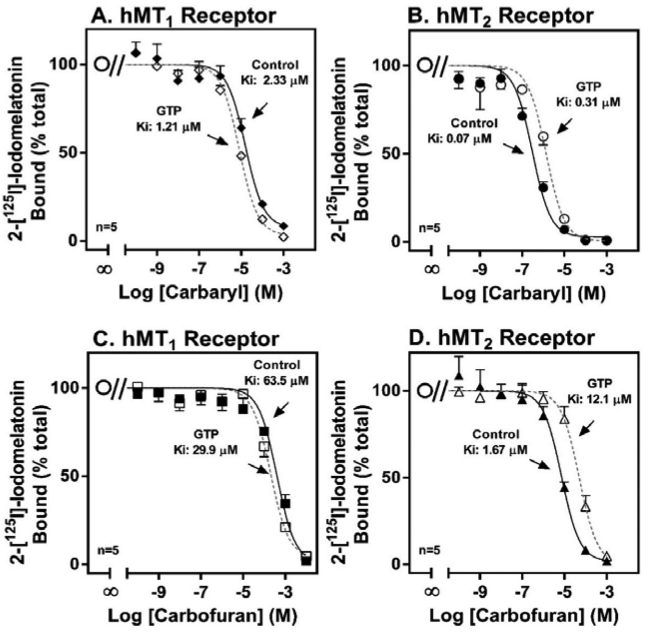

Figure 4. Carbaryl and carbofuran competition for 2-[^{125}I]-iodomelatonin binding to hMT$_1$ and hMT$_2$ melatonin without (control) and with (GTP) G protein inactivation. The ordinate represents 2-[^{125}I]-iodomelatonin binding to either the hMT$_1$ (A,C) or the hMT$_2$ (B,D) melatonin receptors expressed as a percent of total binding. Membranes from CHO cells stably expressing hMT$_1$ and hMT$_2$ melatonin receptors were incubated with 2-[^{125}I]-iodomelatonin (300 pM) in the absence (closed diamond for MT$_1$; closed circle for MT$_2$) and presence (open diamond for MT$_1$; open circle for MT$_2$) of GTP (100 μM) and different concentrations (0.1 nM to 1 mM) of carbaryl (A,B) or carbofuran (C,D). Binding was performed using Tris buffer (Tris-HCl 50 mM and 10 mM $MgCl_2$ at pH 7.4), with the absence or presence of 100 μM GTP, 1 mM EDTA, and 150 mM NaCl, at 25 °C for 1 h. The effect of vehicle (ethanol or DMSO) used to dissolve the carbamate compounds in competition for 2-[^{125}I]-iodomelatonin binding to the melatonin receptors was negligible and hence was not plotted. Data shown are the mean ± SEM of 5−8 independent experiments, expressed as percentage of total binding. Ratio ($K_{iGTP}/K_{iControl}$) refers to the relationship between the K_i values obtained in the presence (K_{iGTP}) and absence ($K_{iControl}$) of GTP.

The absence of a functional group equivalent to melatonin's methoxy-group in the carbamates is probably a significant contributor to their reduced affinity to both melatonin receptors. Participation of histidine (H195 in MT$_1$ and H208 in MT$_2$ or H5.46 in Ballesteros numbering) in hydrogen bonding interactions with melatonergic ligands and as an anchoring residue for receptor−melatonin interactions is implicated using various theoretical homology models of melatonin receptors.[43,45−49] This histidine is in close proximity (2−3 Å), well within the molecular interaction zone to leverage hydrogen bonding and/or hydrophobic interactions, to the bound carbamate ligands in almost all of the top 15

DOI: 10.1021/acs.chemrestox.6b00301
Chem. Res. Toxicol. XXXX, XXX, XXX−XXX

Table 2. Affinity Constants for Carbaryl and Carbofuran Insecticides for Competition for 2-[^{125}I]-Iodomelatonin Binding to hMT$_1$ and hMT$_2$ Melatonin Receptors Expressed in the Absence and Presence of GTP

	2-[^{125}I]-iodomelatonin binding[a]					
	human MT$_1$		ratio[c]	human MT$_2$		ratio[c]
	K_i value (μM)[b]		$K_{iGTP}/K_{iControl}$	K_i value (μM)[b]		$K_{iGTP}/K_{iControl}$
melatonin receptor ligand	control	GTP		control	GTP	
melatonin	$(0.18 \pm 0.03) \times 10^{-3}$	$(0.67 \pm 0.19) \times 10^{-3}$	3.92 ± 0.84	$(0.2 \pm 0.06) \times 10^{-3}$	$(0.4 \pm 0.1) \times 10^{-3}$	2.91 ± 1.30
carbaryl	2.33 ± 0.53	1.21 ± 0.11	0.65 ± 0.16	0.07 ± 0.005	0.31 ± 0.03	4.82 ± 0.82
carbofuran	63.5 ± 12.5	29.9 ± 8.6	0.59 ± 0.24	1.67 ± 0.39	12.1 ± 2.63	10.8 ± 4.2

[a]Competition of carbaryl and carbofuran for 2-[^{125}I]-iodomelatonin binding to CHO cells membranes stably expressing the hMT$_1$ and hMT$_2$ melatonin receptors. Binding was performed at 25 °C for 1 h in the presence (GTP) and absence (control) of 100 μM GTP. [b]K_i values were calculated from IC$_{50}$ values obtained from competition curves using Cheng-Prusoff's equation.[64] K_i values are expressed as the mean ± SEM of 5−6 independent experiments (n = 6 for melatonin, n = 5 for both carbaryl and carbofuran). [c]Ratio MT$_{1GTP}$/MT$_{1Control}$ shows fold difference in the affinity ($K_{iMT1GTP}/K_{iMT2Control}$) of carbaryl and carbofuran for 2-[^{125}I]-iodomelatonin binding to the hMT$_1$ and hMT$_2$ melatonin receptor. Ratios are expressed as the mean ± SEM of 5−10 independent experiments.

conformations sampled for each ligand−receptor docking experiment.

The computational predictions were validated by competition binding of the insecticides for 2-[^{125}I]-iodomelatonin to the hMT$_1$ and hMT$_2$ melatonin receptors. Competition of carbaryl and carbofuran for 2-[^{125}I]-iodomelatonin binding to the hMT$_1$ and hMT$_2$ melatonin receptors was to a single site with 33-fold and 35-fold higher affinity for the hMT$_2$ receptor when compared with that for the hMT$_1$ receptor, respectively. The insecticides compete for either melatonin receptor with lower affinity than melatonin suggesting interaction with a lower affinity melatonin receptor site.[50] Nevertheless, competition by carbamates for 2-[^{125}I]-iodomelatonin binding is within the affinity range observed for other melatonin receptor ligands [e.g., luzindole (K_i hMT$_1$, 179 nM; K_i hMT$_2$, 7.3 nM); 4P-PDOT (K_i hMT$_1$, 658 nM; K_i hMT$_2$, 0.41 nM)] that compete for this radioligand (300 pM).[51,52] Furthermore, these ligands are known to block melatonin signaling and act as inverse agonists at constitutively active hMT$_1$ receptors.[51,52] A recent publication reported that another class of environmental chemicals, phthalate esters, bind with high nanomolar/low micromolar affinity to G protein coupled cannabinoids receptors (CB-1) and mediate functional signaling responses.[53] It follows that carbaryl with an affinity of 2330 nM at hMT$_1$ and 70 nM at MT$_2$ receptors is likely to bind and affect melatonin mediated signaling in target tissues.

A key finding in this study was the carbamate interaction with the two melatonin receptors and their potential to affect signaling. Use of GTP in competition binding assays to assess intrinsic efficacy of G-protein coupled receptor ligands is a well established method to assess a ligand's apparent intrinsic efficacy.[40,41] In this study, we demonstrated that the apparent affinity of carbaryl or carbofuran for 2-[^{125}I]-iodomelatonin binding to the MT$_1$ receptor was not affected, suggesting apparent intrinsic efficacy as neutral antagonists (Figure 4).[40,41] By contrast, the apparent affinities of the two insecticides for the hMT$_2$ melatonin receptor decreased in the presence of GTP, suggesting agonistic apparent efficacy properties (Figure 4).[40,41] These results suggest that exposure to carbamate insecticides that directly bind to human melatonin receptors could either mimic (MT$_2$) or alternatively block (MT$_1$) the effect of endogenous melatonin potentially affecting melatonin receptor mediated signaling in target tissues.

Alternative Mechanism(s) through Which Carbamate Insecticides Could Alter Melatonin Receptor Signaling. In addition to directly binding to human melatonin receptors as demonstrated in our study, the carbamate insecticides carbaryl and carbofuran are reported to alter the metabolism of serotonin (a metabolic precursor of melatonin) and melatonin.[26,54−57] A single oral dose of carbaryl (60 mg/kg) increased the levels of serotonin,[55] and repeated administration of a mixture of parathion, dichlorodiphenyltrichloroethane (DDT), and carbaryl increased the elimination of urinary dopamine and serotonin metabolites 3-methoxy-4-hydroxymandelic acid (VMA) and 5-hydroxy-3-indolyacetic acid (5-HIAA) in mammals.[54]

Carbamate exposure may increase risk for diseases or conditions by (1) altering melatonin production,[56,57] (2) direct action at the level of the MT$_1$ and MT$_2$ melatonin receptors as demonstrated in this study, and/or (3) potentially altering receptor sensitivity and its ability to signal the time of day information at target tissues.[1,18,58−60]

Exposure to environmental chemicals acting via the nuclear transcription factor aryl hydrocarbon receptor and its role in circadian clock and metabolic disruption was recently reported.[61] However, to date the interactions of environmental chemicals with human melatonin receptors have not been reported in the literature. Interactions of carbamates with MT$_1$ and MT$_2$ receptors expressed in the brain (e.g., SCN) and peripheral tissues (e.g., pancreatic β-cells) responding to endogenous melatonin may alter melatonin receptor sensitivity and signaling leading to disruptions in the rhythmic and homeostatic balance of key regulatory processes[1,18,58−60] and metabolic functions.[62] For example, disruption of melatonin receptor signaling in pancreatic β-cells may lead to altered glucose metabolism and insulin release leading to diabetes and metabolic disorders.[2]

The immediate next step is to assess the effect of carbamate insecticides in validated animal models of circadian misalignment to determine whether our *in silico* and *in vitro* findings translate to environmental modulation of melatonin receptor mediated activities *in vivo*. Melatonin xeno-pharmacology is currently under-explored and has the potential of being key for the identification of a new class of environmental neuroendocrine disruptors targeting human melatonin receptors. Assessment of environmental chemicals for their disruption of circadian activity is currently not being considered by Tox21,[63] and designing a comprehensive strategy to the established Chem2Risk linkages for potential environmental circadian disruptors can overcome this limitation. Further, designing studies to specifically assess the risk of exposure to these potential environmental circadian disruptors targeting melato-

DOI: 10.1021/acs.chemrestox.6b00301
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nin receptors will contribute to an increased understanding of and the prevention of diseases and conditions resulting from exposure to these environmental agents.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.chemrestox.6b00301.

Illustrations of theoretical homology models of human melatonin receptors superimposed with human β2-adrenergic receptor (template) and docking poses of melatonin-hMT1 and hMT2 melatonin receptors (PDF)

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Funding

This work was supported by the Jacobs School of Medicine and Biomedical Science funds and USPHS R21 ES023684 to M.L.D. and R.V.R.

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

CHO, Chinese Hamster Ovaries; h, human; SCN, suprachiasmatic nucleus; DSSTox DB, distributed structure-searchable toxicity database; GTP, guanosine triphosphate; CREB, cAMP responsive element-binding protein; cAMP, cyclic adenosine monophosphate; DMSO, dimethyl sulfoxide; EDTA, ethylene diamminetetraacetic acid; AA-NAT, *N*-acetyltransferase; HIOMT, hydroxyindole-*o*-methyltransferase; DSPS, delayed sleep phase syndrome; ASPS, Advanced sleep phase syndrome; SAD, seasonal affective disorders; EPA, Environmental Protection Agency; ACToR, aggregated computational toxicology resource; STITCH, Search Tool for Interactions of Chemicals; CTD, Comparative Toxicogenomics Database; MOE, Molecular Operating Environment; GLIDA, GPCR ligand database

■ REFERENCES

(1) Dubocovich, M. L. (2007) Melatonin receptors: role on sleep and circadian rhythm regulation. *Sleep medicine 8* (Suppl 3), 34−42.

(2) Peschke, E., Bahr, I., and Muhlbauer, E. (2013) Melatonin and pancreatic islets: interrelationships between melatonin, insulin and glucagon. *Int. J. Mol. Sci. 14*, 6981−7015.

(3) Dubocovich, M. L., Delagrange, P., Krause, D. N., Sugden, D., Cardinali, D. P., and Olcese, J. (2010) International Union of Basic and Clinical Pharmacology. LXXV. Nomenclature, classification, and pharmacology of G protein-coupled melatonin receptors. *Pharmacol Rev. 62*, 343−380.

(4) Klein, D. C. (2002) Serotonin N-acetyltransferase. A personal historical perspective. *Adv. Exp. Med. Biol. 460*, 5−16.

(5) Fukuhara, C., Dirden, J. C., and Tosini, G. (2001) Photic regulation of melatonin in rat retina and the role of proteasomal proteolysis. *NeuroReport 12*, 3833−3837.

(6) Gauer, F., and Craft, C. M. (1996) Circadian regulation of hydroxyindole-Omethyltransferase mRNA levels in rat pineal and retina. *Brain Res. 737*, 99−109.

(7) Dubocovich, M. L., Hudson, R. L., Sumaya, I. C., Masana, M. I., and Manna, E. (2005) Effect of MT1 melatonin receptor deletion on melatonin-mediated phase shift of circadian rhythms in the C57BL/6 mouse. *J. Pineal Res. 39*, 113−120.

(8) Bothorel, B., Barassin, S., Saboureau, M., Perreau, S., Vivien-Roels, B., Malan, A., and Pevet, P. (2002) Exogenous melatonin increases the amplitude of the pineal melatonin secretion by a direct action on the circadian clock in the rat. *Eur. J.Neurosci. 16*, 1090−1098.

(9) Zhu, L., and Zee, P. C. (2012) Circadian Rhythm Sleep Disorders. *Neurologic Clinics. 30*, 1167−1191.

(10) Reppert, S. M., Weaver, D. R., and Ebisawa, T. (1994) Cloning and characterization of a mammalian melatonin receptor that mediates reproductive and circadian responses. *Neuron 13*, 1177−1185.

(11) Witt-Enderby, P. A., and Dubocovich, M. L. (1996) Characterization and regulation of the human ML1A melatonin receptor stably expressed in Chinese hamster ovary cells. *Mol. Pharmacol. 50*, 166−174.

(12) Brydon, L., Petit, L., de Coppet, P., Barrett, P., Morgan, P. J., Strosberg, A. D., and Jockers, R. (1999) Polymorphism and signalling of melatonin receptors. *Reprod., Nutr., Dev. 39*, 315−324.

(13) Petit, L., Lacroix, I., de Coppet, P., Strosberg, A. D., and Jockers, R. (1999) Differential signaling of human Mel1a and Mel1b melatonin receptors through the cyclic guanosine 3−5-monophosphate pathway. *Biochem. Pharmacol. 58*, 633−639.

(14) Witt-Enderby, P. A., Masana, M. I., and Dubocovich, M. L. (1998) Physiological exposure to melatonin supersensitizes the cyclic adenosine 3′,5′-monophosphate-dependent signal transduction cascade in Chinese hamster ovary cells expressing the human mt1 melatonin receptor. *Endocrinology 139*, 3064−3071.

(15) Hunt, A. E., Al-Ghoul, W. M., Gillette, M. U., and Dubocovich, M. L. (2001) Activation of MT(2) melatonin receptors in rat suprachiasmatic nucleus phase advances the circadian clock. *Am. J. Physiol. Cell Physiol. 280*, C110−118.

(16) Witt-Enderby, P. A., MacKenzie, R. S., McKeon, R. M., Carroll, E. A., Bordt, S. L., and Melan, M. A. (2000) Melatonin induction of filamentous structures in non-neuronal cells that is dependent on expression of the human mt1 melatonin receptor. *Cell Motil. Cytoskeleton 46*, 28−42.

(17) Nelson, C. S., Marino, J. L., and Allen, C. N. (1996) Melatonin receptors activate heteromeric G-protein coupled Kir3 channels. *NeuroReport 7*, 717−720.

(18) Liu, J., Clough, S. J., Hutchinson, A. J., Adamah-Biassi, E. B., Popovska-Gorevski, M., and Dubocovich, M. L. (2016) MT1 and MT2Melatonin Receptors: A Therapeutic Perspective. *Annu. Rev. Pharmacol. Toxicol. 56*, 361−383.

(19) Blask, D. E., Brainard, G. C., Dauchy, R. T., Hanifin, J. P., Davidson, L. K., Krause, J. A., Sauer, L. A., Rivera-Bermudez, M. A., Dubocovich, M. L., Jasser, S. A., Lynch, D. T., Rollag, M. D., and Zalatan, F. (2005) Melatonin-depleted blood from premenopausal women exposed to light at night stimulates growth of human breast cancer xenografts in nude rats. *Cancer Res. 65*, 11174−11184.

(20) Peschke, E. (2008) Melatonin, endocrine pancreas and diabetes. *J. Pineal Res. 44*, 26−40.

(21) Krause, D. N., Geary, G. G., Doolen, S., and Duckles, S. P. (2002) Melatonin and cardiovascular function. *Adv. Exp. Med. Biol. 460*, 299−310.

(22) Kobayashi, H., Yuyama, A., Kajita, T., Shimura, K., Ohkawa, T., and Satoh, K. (1985) Effects of insecticidal carbamates on brain acetylcholine content, acetylcholinesterase activity and behavior in mice. *Toxicol. Lett. 29*, 153−159.

(23) Wesseling, C., de Joode, V. B., Keifer, M., London, L., Mergler, D., and Stallones, L. (2010) Symptoms of psychological distress and suicidal ideation among banana workers with a history of poisoning by organophosphate or n-methyl carbamate pesticides. *Occup. Environ. Med. 67*, 778−784.

DOI: 10.1021/acs.chemrestox.6b00301
Chem. Res. Toxicol. XXXX, XXX, XXX−XXX

(24) Beard, J. D., Hoppin, J. A., Richards, M., Alavanja, M. C. R., Blair, A., Sandler, D. P., and Kamelb, F. (2013) Pesticide Exposure and Self-Reported Incident Depression among Wives in the Agricultural Health Study. *Environ. Res. 126*, 31−42.
(25) Montgomery, M. P., Kamel, F., Saldana, T. M., Alavanja, M. C., and Sandler, D. P. (2008) Incident diabetes and pesticide exposure among licensed pesticide applicators: Agricultural Health Study, 1993−2003. *Am. J. Epidemiol. 167*, 1235−1246.
(26) Hassan, A. (1971) Pharmacological effects of carbaryl. I. The effect of carbaryl on the synthesis and degradation of catecholamines in the rat. *Biochem. Pharmacol. 20*, 2299−2308.
(27) Ahdaya, S. M., Shah, P. V., and Guthrie, F. E. (1976) Thermoregulation in mice treated with parathion, carbaryl, or DDT. *Toxicol. Appl. Pharmacol. 35*, 575−580.
(28) Moser, V. C., Phillips, P. M., and McDaniel, K. L. (2015) Assessment of biochemical and behavioral effects of carbaryl and methomyl in Brown-Norway rats from preweaning to senescence. *Toxicology 331*, 1−13.
(29) Gaulton, A., Bellis, L. J., Bento, A. P., Chambers, J., Davies, M., Hersey, A., Light, Y., McGlinchey, S., Michalovich, D., Al-Lazikani, B., and Overington, J. P. (2012) ChEMBL: a large-scale bioactivity database for drug discovery. *Nucleic Acids Res. 40*, D1100−1107.
(30) Kuhn, M., Szklarczyk, D., Franceschini, A., Campillos, M., von Mering, C., Jensen, L. J., Beyer, A., and Bork, P. (2010) STITCH 2: an interaction network database for small molecules and proteins. *Nucleic Acids Res. 38*, D552−556.
(31) Davis, A. P., Murphy, C. G., Johnson, R., Lay, J. M., Lennon-Hopkins, K., Saraceni-Richards, C., Sciaky, D., King, B. L., Rosenstein, M. C., Wiegers, T. C., and Mattingly, C. J. (2013) The Comparative Toxicogenomics Database: update 2013. *Nucleic Acids Res. 41*, D1104−1114.
(32) Chen, X., and Reynolds, C. H. (2002) Performance of similarity measures in 2D fragment-based similarity searching: comparison of structural descriptors and similarity coefficients. *Journal of chemical information and computer sciences 42*, 1407−1414.
(33) Backman, T. W., Cao, Y., and Girke, T. (2011) ChemMine tools: an online service for analyzing and clustering small molecules. *Nucleic Acids Res. 39*, W486−491.
(34) Eswar, N., Webb, B., Marti-Renom, M. A., Madhusudhan, M. S., Eramian, D., Shen, M. Y., Pieper, U., and Sali, A. (2007) Comparative protein structure modeling using MODELLER. *Curr. Protoc. Protein Sci.*, Chapter 2, Unit 2.9, p 2.9.1, DOI: 10.1002/0471140864.ps0209s50.
(35) Akula, N., and Pattabiraman, N. (2005) A systematic search method for the identification of tightly packed transmembrane parallel alpha-helices. *J. Biomol. Struct. Dyn. 22*, 625−634.
(36) Okuno, Y., Tamon, A., Yabuuchi, H., Niijima, S., Minowa, Y., Tonomura, K., Kunimoto, R., and Feng, C. (2008) GLIDA: GPCR–ligand database for chemical genomics drug discovery–database and tools update. *Nucleic Acids Res. 36*, D907−912.
(37) Pettersen, E. F., Goddard, T. D., Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C., and Ferrin, T. E. (2004) UCSF Chimera–a visualization system for exploratory research and analysis. *J. Comput. Chem. 25*, 1605−1612.
(38) Reppert, S. M., Godson, C., Mahle, C. D., Weaver, D. R., Slaugenhaupt, S. A., and Gusella, J. F. (1995) Molecular characterization of a second melatonin receptor expressed in human retina and brain: the Mel1b melatonin receptor. *Proc. Natl. Acad. Sci. U. S. A. 92*, 8734−8738.
(39) Gerdin, M. J., Masana, M. I., Ren, D., Miller, R. J., and Dubocovich, M. L. (2003) Short-term exposure to melatonin differentially affects the functional sensitivity and trafficking of the hMT1 and hMT2 melatonin receptors. *J. Pharmacol. Exp. Ther. 304*, 931−939.
(40) Lefkowitz, R. J., Mullikin, D., and Caron, M. G. (1976) Regulation of beta-adrenergic receptors by guanyl-5′-yl imidodiphosphate and other purine nucleotides. *J. Biol. Chem. 251*, 4686−4692.
(41) Noël, F., Pompeu, T. E., and Moura, B. C. (2014) Functional binding assays for estimation of the intrinsic efficacy of ligands at the 5-HT1A receptor: application for screening drug candidates. *J. Pharmacol. Toxicol. Methods 70*, 12−18.
(42) Gupta, R. C. (1994) Carbofuran toxicity. *J. Toxicol. Environ. Health 43*, 383−418.
(43) Pala, D., Beuming, T., Sherman, W., Lodola, A., Rivara, S., and Mor, M. (2013) Structure-based virtual screening of MT2 melatonin receptor: influence of template choice and structural refinement. *J. Chem. Inf. Model. 53*, 821−835.
(44) Rivara, S., Lorenzi, S., Mor, M., Plazzi, P. V., Spadoni, G., Bedini, A., and Tarzia, G. (2005) Analysis of structure-activity relationships for MT2 selective antagonists by melatonin MT1 and MT2 receptor models. *J. Med. Chem. 48*, 4049−4060.
(45) Jockers, R., Delagrange, P., Dubocovich, M. L., Markus, R. P., Renault, N., Tosini, G., Cecon, E., and Zlotos, D. P. (2016) Update on melatonin receptors: IUPHAR Review. *Br. J. Pharmacol. 173*, 2702−2725.
(46) Mazna, P., Obsilova, V., Jelinkova, I., Balik, A., Berka, K., Sovova, Z., Ettrich, R., Svoboda, P., Obsil, T., and Teisinger, J. (2004) Molecular modeling of human MT2 melatonin receptor: the role of Val204, Leu272 and Tyr298 in ligand binding. *J. Neurochem. 91*, 836−842.
(47) Farce, A., Chugunov, A. O., Loge, C., Sabaouni, A., Yous, S., Dilly, S., Renault, N., Vergoten, G., Efremov, R. G., Lesieur, D., and Chavatte, P. (2008) Homology modeling of MT1 and MT2 receptors. *Eur. J. Med. Chem. 43*, 1926−1944.
(48) Pala, D., Lodola, A., Bedini, A., Spadoni, G., and Rivara, S. (2013) Homology models of melatonin receptors: challenges and recent advances. *Int. J. Mol. Sci. 14*, 8093−8121.
(49) Rivara, S., Pala, D., Lodola, A., Mor, M., Lucini, V., Dugnani, S., Scaglione, F., Bedini, A., Lucarini, S., Tarzia, G., and Spadoni, G. (2012) MT1-selective melatonin receptor ligands: synthesis, pharmacological evaluation, and molecular dynamics investigation of N-{[(3-O-substituted)anilino]alkyl}amides. *ChemMedChem 7*, 1954−1964.
(50) Loudon, M., and Zisapel, N. (1986) Characterization of central melatonin receptors using 125I-melatonin. *FEBS Lett. 197*, 9−12.
(51) Dubocovich, M. L., Yun, K., Al-Ghoul, W. M., Benloucif, S., and Masana, M. I. (1998) Selective MT2 melatonin receptor antagonists block melatonin-mediated phase advances of circadian rhythms. *FASEB J. 12*, 1211−1220.
(52) Beresford, I. J., Browning, C., Starkey, S. J., Brown, J., Foord, S. M., Coughlan, J., North, P. C., Dubocovich, M. L., and Hagan, R. M. (1998) GR196429: a nonindolic agonist at high-affinity melatonin receptors. *J. Pharmacol. Exp. Ther. 285*, 1239−1245.
(53) Bisset, K. M., Dhopeshwarkar, A. S., Liao, C., and Nicholson, R. A. (2011) The G protein-coupled cannabinoid-1 (CB1) receptor of mammalian brain: inhibition by phthalate esters in vitro. *Neurochem. Int. 59*, 706−713.
(54) Hassan, A., and Cueto, C., Jr. (1970) Biochemical effects in the rabbit of repeated administration of a mixture of DDT, carbaryl, and parathion. *Z. Naturforsch., B: J. Chem. Sci. 25*, 521−525.
(55) Hassan, A., and Santolucito, J. A. (1971) Pharmacological effects of carbaryl. II. Modification of serotonin metabolism in the rat brain. *Experientia 27*, 287−288.
(56) Attia, A. M., Reiter, R. J., Nonaka, K. O., Mostafa, M. H., Soliman, S. A., and el-Sebae, A. H. (1991) Carbaryl-induced changes in indoleamine synthesis in the pineal gland and its effects on nighttime serum melatonin concentrations. *Toxicology 65*, 305−314.
(57) Attia, A. M., Reiter, R. J., Withyachumnarnkul, B., Mostafa, M. H., Soliman, S. A., and el-sebae, A. K. (1991) Chronic administration of sublethal doses of carbaryl increases pineal N-acetyltransferase and hydroxyindole-O-methyltransferase activities and serum melatonin levels. *J. Pineal Res. 10*, 49−54.
(58) Gerdin, M. J., Masana, M. I., and Dubocovich, M. L. (2004) Melatonin-mediated regulation of human MT(1) melatonin receptors expressed in mammalian cells. *Biochem. Pharmacol. 67*, 2023−2030.
(59) Gerdin, M. J., Masana, M. I., Rivera-Bermudez, M. A., Hudson, R. L., Earnest, D. J., Gillette, M. U., and Dubocovich, M. L. (2004) Melatonin desensitizes endogenous MT2 melatonin receptors in the rat suprachiasmatic nucleus: relevance for defining the periods of

DOI: 10.1021/acs.chemrestox.6b00301
Chem. Res. Toxicol. XXXX, XXX, XXX−XXX

sensitivity of the mammalian circadian clock to melatonin. *FASEB J. 18*, 1646−1656.
(60) Tuomi, T., Nagorny, C. L., Singh, P., Bennet, H., Yu, Q., Alenkvist, I., Isomaa, B., Ostman, B., Soderstrom, J., Pesonen, A. K., Martikainen, S., Raikkonen, K., Forsen, T., Hakaste, L., Almgren, P., Storm, P., Asplund, O., Shcherbina, L., Fex, M., Fadista, J., Tengholm, A., Wierup, N., Groop, L., and Mulder, H. (2016) Increased Melatonin Signaling Is a Risk Factor for Type 2 Diabetes. *Cell Metab. 23*, 1067.
(61) Jaeger, C., and Tischkau, S. A. (2016) Role of Aryl Hydrocarbon Receptor in Circadian Clock Disruption and Metabolic Dysfunction. *Environ. Health Insights 10*, 133−141.
(62) Peschke, E., and Peschke, D. (1998) Evidence for a circadian rhythm of insulin release from perifused rat pancreatic islets. *Diabetologia 41*, 1085−1092.
(63) Kavlock, R. J., Austin, C. P., and Tice, R. (2009) Toxicity Testing in the 21st Century: Implications for Human Health Risk Assessment. *Risk Anal. 29*, 485−497.
(64) Cheng, Y.-C., and Prusoff, W. H. (1973) Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50% inhibition (I50) of an enzymatic reaction. *Biochem. Pharmacol. 22*, 3099−3108.

DOI: 10.1021/acs.chemrestox.6b00301
Chem. Res. Toxicol. XXXX, XXX, XXX−XXX