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Carbamate Insecticides Target Human Melatonin Receptors

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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 MT₁ and MT₂ melatonin receptors *in silico*. Carbaryl and carbofuran then were tested for competition with 2-[¹²⁵I]-iodomelatonin (300 pM) binding to hMT₁ or hMT₂ receptors stably expressed in CHO cells. Carbaryl and carbofuran showed higher affinity for competition with 2-[¹²⁵I]-iodomelatonin binding to the hMT₂ compared to the hMT₁ 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-[¹²⁵I]-iodomelatonin binding for the hMT₁ melatonin receptor was not affected but significantly decreased for the hMT₂ 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.

Graphical abstract

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.chemres-tox.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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Notes

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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 MT₁ and/or MT₂.¹⁻³ Rhythmicity of pineal melatonin synthesis from serotonin is tightly orchestrated by *N*-acetyltransferase (AA-NAT) and hydroxyindole- α -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 signaling³ 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 knowledge-base. Molecular docking of select

environmental modulators established novel bimolecular interactions of carbamate insecticides carbaryl and carbofuran with the MT₁ and MT₂ melatonin receptors *in silico*. Furthermore, the current study investigated ligand affinity, selectivity, and apparent intrinsic efficacy of selected environmental disruptors on hMT₁ and hMT₂ 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 tremor²⁶ and hypothermia.²⁷ Furthermore, a recent study reports age-specific 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 hMT₁ (350 residues) and hMT₂ (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 β_2 AR) has 34% and 32% sequence similarity with hMT₁ and hMT₂ melatonin receptors, respectively. The high resolution X-ray crystal structure of h β_2 AR (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 MT₁ and MT₂, respectively. Several theoretical models of MT₁ and MT₂ were generated using Modeller9v8.³⁴ The putative binding pockets for MT₁ (inclusive of residues H205, S110, and S112) and for MT₂ (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 MT₁ and MT₂ 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 MT₁ and MT₂ 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 hMT₁ (human Mel_{1a} cloned into pcDNA1) and hMT₂ 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-[¹²⁵I]-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 MgCl₂ 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-[¹²⁵I]-iodomelatonin binding when tested alone.

FLAG Tagging and Stable Transfection

The development of CHO-hMT₁-FLAG and CHO-hMT₂-FLAG stably expressing the human MT₁ and MT₂ 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 hMT₁-GFP or hMT₂-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% CO₂ at 37 °C. CHO-MT₁ and CHO-MT₂ 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-[¹²⁵I]-Iodomelatonin Binding Studies

Cell pellets were resuspended in 50 mM Tris-HCl (pH 7.4; 10 mM MgCl₂), homogenized, pelleted, and washed by centrifugation (20,000–30,000g). 2-[¹²⁵I]-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 MgCl₂), 2-[¹²⁵I]-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 MgCl₂, pH 7.4, to yield approximately 6–16 µg of protein/assay. 2-[¹²⁵I]-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 MgCl₂, 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 interface³³ resulted in 71 compounds clustered with melatonin including hydroxychalcones, flavones, tamoxifen, carbaryl, and carbofuran (highlighted in gray, Figure 1). A majority of the 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 activity⁴² 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 hMT₁ and hMT₂ Melatonin Receptor Binding Sites

Melatonin and the carbamates were scored after docking into the putative melatonin binding pockets in hMT₁ and hMT₂ receptor models. Melatonin docks into the putative melatonin binding site with H195 (in hMT₁) or H208 (in hMT₂) 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–hMT₂ receptor complexes were melatonin (11 ± 0.6) > carbaryl (8.7 ± 0.5) > carbofuran (7.9 ± 0.2), whereas the corresponding scores for the ligand–hMT₁ 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–MT₂ complexes were –7.7 (carbaryl) and –5.8 (carbofuran) compared to –11.5 for the melatonin–MT₂ complex consistent with the results of surflex docking scores.

Competition of Carbamates for 2-[¹²⁵I]-Iodomelatonin Binding to Human Melatonin Receptors

The affinities of both carbaryl and carbofuran for competition to 2-[¹²⁵I]-iodomelatonin binding were determined for both hMT₁ and hMT₂ melatonin receptors stably expressed in CHO cells. Carbaryl (10 nM–1 mM) and carbofuran (10 nM–10 mM) competed with 2-[¹²⁵I]-iodomelatonin binding (300 pM) to membranes from CHO cells stably expressing either human recombinant hMT₁ or hMT₂ melatonin receptors⁴⁰ (Figure 3C and F). Table 1 shows affinity constants (K_i values) for the competition of melatonin, carbaryl, and carbofuran for 2-[¹²⁵I]-iodomelatonin binding to the hMT₁ and hMT₂ melatonin receptors. The affinity of carbaryl for the hMT₂ receptor was 33-fold higher than the hMT₁ melatonin receptor, while carbofuran showed a 35-fold difference in binding affinity between the hMT₁ and hMT₂ receptors (Table 1; Figure 3C,F). Both pesticides showed selectivity for hMT₂ over hMT₁; however, carbaryl showed higher affinity for either the hMT₁ or the hMT₂ receptors when compared with that for carbofuran (Table 1). We should note that the affinity of melatonin competition for 2-[¹²⁵I]-iodomelatonin was 8,100 and 233 times higher than that for carbaryl on the hMT₁ and hMT₂ melatonin receptors, respectively (Table 1).

Competition of Carbaryl and Carbofuran for 2-[¹²⁵I]-Iodomelatonin Binding to CHO-hMT₁ and CHO-hMT₂ 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 μ M) during competition with 2-[¹²⁵I]-iodomelatonin binding to hMT₁ and hMT₂ 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 hMT₁, while it significantly decreased it for the hMT₂ (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 hMT₁ (Figure 4A and Table 2), while the ratio $K_{iGTP}/K_{iControl}$ for that of hMT₂ 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 hMT₁ melatonin receptor was not affected in the presence of GTP, showing a ratio [$K_{iGTP}/K_{iControl} = 0.59 \pm 0.24$] (Figure 4C, Table 2) compatible with those expected for an antagonist/inverse agonist. However, GTP significantly decreased the apparent affinity of carbofuran for the hMT₂ receptor suggesting agonist efficacy ($K_{iGTP}/K_{iControl} = 10.8 \pm 4.2$) (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 hMT₁ and hMT₂ receptor models with higher affinity for the hMT₂ receptors, which was verified in competition studies of 2-[¹²⁵I]-iodomelatonin binding to the human melatonin receptors. Lastly, both carbamates show distinct apparent

intrinsic efficacy to the hMT₁ and MT₂ 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 hMT₁ or hMT₂ bound carbamate insecticides were 20–30% lower than that of melatonin. While the cognate ligand melatonin shows no preference toward hMT₁ or hMT₂, both carbamate ligands showed greater preference to hMT₂ receptor over hMT₁. The enhanced binding of the carbamate pesticides with hMT₂ 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 MT₂ antagonist UCM454 and the selective partial agonist acylaminoethyl tetralin's interactions with hMT₂ receptors.^{43,44} The carbamate insecticides carbaryl and carbofuran, docked very similarly to the putative binding modes suggested for prototype melatonergic ligands with hMT₂ receptors⁴⁵ 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.

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 MT₁ and H208 in MT₂ 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 conformations sampled for each ligand–receptor docking experiment.

The computational predictions were validated by competition binding of the insecticides for 2-[¹²⁵I]-iodomelatonin to the hMT₁ and hMT₂ melatonin receptors. Competition of carbaryl and carbofuran for 2-[¹²⁵I]-iodomelatonin binding to the hMT₁ and hMT₂ melatonin receptors was to a single site with 33-fold and 35-fold higher affinity for the hMT₂ receptor when compared with that for the hMT₁ 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-[¹²⁵I]-iodomelatonin binding is within the affinity range observed for other melatonin receptor ligands [e.g., luzindole (K_i hMT₁, 179 nM; K_i hMT₂, 7.3 nM); 4P-PDOT (K_i hMT₁, 658 nM; K_i hMT₂, 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 hMT₁ 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 hMT₁ and 70 nM at MT₂ 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-[¹²⁵I]-iodomelatonin binding to the MT₁ 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 hMT₂ 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 (MT₂) or alternatively block (MT₁) 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-indolylacetic 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 MT₁ and MT₂ 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 MT₁ and MT₂ 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 melatonin receptors will contribute to an increased understanding of and the prevention of diseases and conditions resulting from exposure to these environmental agents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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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	<i>N</i> -acetyltransferase
HIOMT	hydroxyindole- <i>o</i> -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

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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.

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-[¹²⁵I]-iodomelatonin binding to the hMT₁ and hMT₂ melatonin receptors. Binding poses of carbaryl-hMT₁ (A) hMT₂ (B) or carbofuran-hMT₁ (D) and hMT₂ complexes (E). The ordinate represents 2-[¹²⁵I]-iodomelatonin binding to either the hMT₁ or hMT₂ receptors expressed as percent of total binding (C). Membranes from CHO cells stably expressing hMT₁ and hMT₂ receptors were incubated with 2-[¹²⁵I]-iodomelatonin (300 pM) in the absence (open circle) and presence (closed diamond for MT₁; closed circle for MT₂) of different concentrations of carbaryl (0.1 nM to 1 mM). The ordinate represents 2-[¹²⁵I]-iodomelatonin binding to either the hMT₁ or hMT₂ melatonin receptors expressed as percent of total binding (F). Membranes from CHO cells stably expressing hMT₁ and hMT₂ melatonin receptors were incubated with 2-[¹²⁵I]-iodomelatonin (300 pM) in the absence (open circle) and presence (closed square for MT₁; closed triangle for MT₂) 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-[¹²⁵I]-iodomelatonin binding to the hMT₁ and hMT₂ was negligible and hence was not plotted. Values are the mean ± SEM of 8 independent experiments.

Figure 4.

Carbaryl and carbofuran competition for 2-[¹²⁵I]-iodomelatonin binding to hMT₁ and hMT₂ melatonin without (control) and with (GTP) G protein inactivation. The ordinate represents 2-[¹²⁵I]-iodomelatonin binding to either the hMT₁ (A,C) or the hMT₂ (B,D) melatonin receptors expressed as a percent of total binding. Membranes from CHO cells stably expressing hMT₁ and hMT₂ melatonin receptors were incubated with 2-[¹²⁵I]-iodomelatonin (300 pM) in the absence (closed diamond for MT₁; closed circle for MT₂) and presence (open diamond for MT₁; open circle for MT₂) 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 MgCl₂ 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-[¹²⁵I]-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.

Table 1

Affinity Constants for Melatonin, Carbaryl, and Carbofuran Insecticides for Competition with 2-[¹²⁵I]-Iodomelatonin Binding to hMT₁ and hMT₂ Melatonin Receptors Expressed in CHO Cells

melatonin receptor ligands	2-[¹²⁵ I]-iodomelatonin binding		
	hMT ₁ ^a	hMT ₂ ^b	ratio ^c
	<i>K_i</i> value (μM)	<i>K_i</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-[¹²⁵I]-iodomelatonin binding to CHO cell membranes stably expressing the hMT₁ and hMT₂ melatonin receptors. Binding was performed at 25 °C for 1 h.

^b *K_i* values were calculated from IC₅₀ 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 (MT₁), *n* = 6 for melatonin (MT₂), *n* = 8 for both carbaryl and carbofuran).

^c Ratio MT₁/MT₂ shows fold difference in affinity (*K_i*_{MT1}/*K_i*_{MT2}) of carbaryl and carbofuran for 2-[¹²⁵I]-iodomelatonin binding to the human MT₁ and MT₂ melatonin receptor.

Affinity Constants for Carbaryl and Carbofuran Insecticides for Competition for 2-[¹²⁵I]-Iodomelatonin Binding to hMT₁ and hMT₂ Melatonin Receptors Expressed in the Absence and Presence of GTP

		2-[¹²⁵ I]-iodomelatonin binding ^a			
		human MT ₁		human MT ₂	
		ratio ^c		ratio ^c	
		K_i value (μ M) ^b		K_i value (μ M) ^b	
		$K_{iGTP}/K_{iControl}$		$K_{iGTP}/K_{iControl}$	
melatonin receptor ligand		control	GTP	control	GTP
melatonin		(0.18 ± 0.03) × 10 ⁻³	(0.67 ± 0.19) × 10 ⁻³	3.92 ± 0.84	(0.2 ± 0.06) × 10 ⁻³
carbaryl		2.33 ± 0.53	1.21 ± 0.11	0.65 ± 0.16	0.31 ± 0.03
carbofuran		63.5 ± 12.5	29.9 ± 8.6	0.59 ± 0.24	12.1 ± 2.63
					10.8 ± 4.2

^a Competition of carbaryl and carbofuran for 2-[¹²⁵I]-iodomelatonin binding to CHO cells membranes stably expressing the hMT₁ and hMT₂ melatonin receptors. Binding was performed at 25 °C for 1 h in the presence (GTP) and absence (control) of 100 μ M GTP.

^b K_i values were calculated from IC₅₀ 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 MT₁ GTP/MT₁Control shows fold difference in the affinity ($K_{iMT1GTP}/K_{iMT2Control}$) of carbaryl and carbofuran for 2-[¹²⁵I]-iodomelatonin binding to the hMT₁ and hMT₂ melatonin receptor. Ratios are expressed as the mean ± SEM of 5–10 independent experiments.