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REVIEW

Modeling defensive functions of alkaloids within diverse chemical portfolios

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Abstract

Studies of the evolution of anti-predatory phenotypes such as aposematic signals have proven informative to demonstrate the role of selection on phenotypic divergence. *Oophaga pumilio* show high variance in both elements of their aposematic signals; visual cues consisting of color patterns, as well as their alkaloid chemical defenses where an individual frog can possess dozens of alkaloid types. Disentangling the function of individual alkaloids is therefore complicated due to various modes of chemical defenses, making simple comparisons between levels of relative 'toxicity' between populations difficult until we can elucidate the defensive capabilities of alkaloids. In this study we model binding affinity of the most abundant alkaloids found in divergent populations of *O. pumilio* which we discovered had a high affinity for binding with Muscarinic acetylcholine receptors in various potential predator classes. Molecular interaction and docking experiments indicate that interactions between alkaloid and muscarinic receptors are highly conserved, and muscarinic receptors themselves show evidence of strong purifying selection. Therefore, we predict functional redundancy is plausible among the most common alkaloids against common targets, and these alkaloids likely function similarly across diverse suites of predators. This affords a predictable baseline of defenses for this combination of alkaloids and receptors between divergent populations which vary in aposematic signals.

Keywords Poison frogs · *Oophaga pumilio* · Alkaloids · Modeling · Defenses · Defensive function

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Introduction

Chemical defenses are widespread and help to render prey species unpalatable (Bolton et al. 2017; Winters et al. 2018, 2021; Lawrence et al. 2019), increase toxicity (Maan and Cummings 2012) or impose enough fitness costs to predators (Marples et al. 2018) to encourage them to consider alternative prey items (Schulte et al. 2017). Some species achieve this with few highly potent or potentially lethal defensive compounds (e.g. tetrodotoxin or batrachotoxin: Daly 1995; Hanifin 2010; Santos et al. 2016, rattlesnake venoms: Robinson et al. 2021). Alternatively, other species can gain sufficient protection using larger chemical portfolios comprised of non-lethal alkaloids (Santos and Cannatella 2011). To date over 1200 alkaloids have been identified in the skins of amphibian species such as poison frogs (Hovey et al. 2018) representing substantial diversity in defensive compounds. High diversity within frog alkaloid profiles has even been found on quite fine scales, including intrapopulation variance (Saporito et al. 2009; Lawrence et al. 2023), and even temporal differences within a single population of poison frogs (Saporito et al. 2006, 2007, 2009). Variation in plant chemical defenses is common and predictions related to how herbivore predators respond to variation in defenses is comparatively more advanced (Wetzel and Whitehead 2020) than our understanding of variation in aposematic prey defenses and their effect on defensive functions in animals (Lawrence et al. 2019; Hämäläinen et al. 2020; Ottocento et al. 2022).

Chemically defended aposematic species advertise their toxicity using identifiable, memorable signals (Mappes et al. 2005). In visual ecology, modeling approaches approximate the perception of diverse species which are widely employed to estimate how signals like aposematic coloration would be perceived. These methods have greatly improved our understanding of if, and how, viewers are able to detect even subtle variation in prey species warning coloration (Crothers and Cummings 2013; Flores et al. 2013; Richards-Zawacki et al. 2013; Barnett et al. 2018; Yeager et al. 2022). However, equivalent modeling techniques are not commonly implemented to determine whether diversity in chemical portfolios changes defensive functions among classes of would-be predators. Inspired by these visual models, we attempt a similar broad approach to model the binding potential of non-lethal frog alkaloids with target receptors across broad potential predator classes, much like visual models are used for estimating visual perception.

Studies of alkaloid function are often limited in scope to single or few alkaloids (Daly et al. 2003; but see: Lawrence et al. 2023) and with few exceptions (e.g. Rojas et al. 2017) generally restricted to laboratory animals, rather than potentially relevant classes of natural predator, which has raised criticisms over the validity of interpreting toxicity metrics derived from these methods (Weldon 2017). Therefore, comparing the levels of defense between populations or species is difficult and potentially incomplete in scope. Computational modeling which simulates interactions with a relevant predator class provides a promising path forward, and may assuage concerns over biological relevance. Although not a direct measure of an alkaloids function in a specific predator, it would still afford us the capability to more efficiently and comprehensively predict, and/or isolate specific potential defensive functions of individual compounds.

In this study we aim to identify possible alkaloid binding targets that are present among diverse taxa of potential predator classes, including influencing possible intraspecific effects such autotoxicity (Santos et al. 2016; Tarvin et al. 2016; Abderemane-Ali et al. 2021). We study the defensive functions of *O. pumilio*, a polytypic species which displays considerable geographic variation in color patterns (Wang and Summers 2010; Hauswaldt

et al. 2011; Richards-Zawacki et al. 2013; Yang et al. 2019; Yeager et al. 2023). Rapid divergence in aposematic coloration has provoked considerable interest in understanding the evolutionary dynamics which has produced this variation (e.g. Summers et al. 1999; Rudh et al. 2007, 2011; Richards-Zawacki et al. 2012; Cummings and Crothers 2013; Yeager et al. 2023, among others). As advances are made in understanding the evolution of the color pattern components of aposematic signal, we would likewise benefit from understanding how the defensive role of chemical defenses vary geographically with warning signals (e.g. Darst et al. 2006; Wang 2011; Maan and Cummings 2012; McGugan et al. 2016; Santos et al. 2016). Here we compare common alkaloids across four phenotypically divergent populations. Dendrobatid poison frogs cannot manufacture their own chemical defenses and must acquire and accumulate them from exogenous sources such as invertebrate prey (Daly et al. 2000; Saporito et al. 2009; Santos and Cannatella 2011), where in many cases their defensive portfolios are extensive (Daly et al. 2005; Saporito et al. 2007). In these poison frogs aposematism is thought to have evolved multiple times, often concurrently with dietary specialization so as to specialize in prey which are sources of defensive alkaloids (Santos et al. 2003; Darst et al. 2005; Santos and Cannatella 2011).

Until the functions of more alkaloid classes are revealed, it remains unclear how alkaloid quantity and diversity relate to defensive capabilities (Lawrence et al. 2019). Even more nebulous is the relationship between overall chemical profiles and divergent aposematic phenotypes, particularly within or between populations of polymorphic/polytypic species such as *O. pumilio*. On one hand, alkaloids could have divergent defensive functions by targeting specific receptors, or perhaps be predator-class specific (Rojas et al. 2017). Alternatively, general defensive function could be highly conserved (therefore making alkaloids functionally redundant) which would ensure defensive function (although alkaloid quantities may also be variable), and potentially honest signaling despite compositional differences in chemical profiles. We test these two alternative hypotheses related to alkaloid diversity by first identifying binding targets for different abundant alkaloids, and then performing molecular interaction and docking experiments to quantify this binding potential. Our analyses highlight a subset of the 17 most common alkaloids which were recovered from a previous study which represents four highly phenotypically diverse adjacent populations of the poison frog *O. pumilio* including red, orange, yellow/green and dark blue populations (Yeager 2015).

Materials and methods

Overview of methods

Alkaloids were first recovered and identified from frog skins as part of previous studies (Yeager 2015). We then identified potential targets for the most common alkaloids. Considering that computational target fishing algorithms are biased in favor of human ligand-receptor interactions, only *Homo sapiens* proteins were considered for consensus predictions. Homology analyses between the most relevant targets were conducted to include additional species which broadly represent conspecifics and heterospecifics (such as potential avian predators). After consensus strategy, we selected a relevant group of protein targets for further analysis using molecular docking and molecular dynamics simulations. The potential binding of the studied alkaloids was evaluated for the most relevant receptors from other non-toxic frog genomes *Nanorana parkeri*, *Xenopus laevis*, *Xenopus tropicalis* as well as chickens (*Gallus*

gallus, representing potential avian predators) and *Homo sapiens*. The final criterion for evaluating the alkaloid-receptor interaction strength was computing the free energy of binding from the molecular dynamic simulations, to quantify binding potentials.

Alkaloid profiles

Frog alkaloids were sampled throughout the Bocas del Toro archipelago and adjacent mainland of Panama in June and July of 2011 and 2012 as part of a complementary study (Yeager 2015; Yeager, McGraw, Saporito, Owens, Giltz and Richards-Zawacki *unpublished data*, Table S1). Four phenotypically distinct populations were chosen encompassing a continuum of conspicuousness (to human viewers) from dull (Aguacate Peninsula, dark blue), intermediate (Isla Colon, yellow/green with black spots and light blue legs) and more highly contrasting (Solarte, orange; Almirante, red with blue legs) aposematic phenotypes. Alkaloids were extracted following Crothers et al. (2016), for full specifics see Yeager (2015). Our work utilizes a reduced version of that published dataset, composed of the 17 most abundant alkaloids reported in that study which represents approximately 73% of the total quantity of chemical defenses across populations (Fig. 1).

Prediction of potential interactions between frog alkaloids and target proteins

We combined two strategies in order to predict possible protein targets: (1) in silico prediction of alkaloid-protein interactions using a consensus strategy, and (2) study of the interaction between alkaloids and selected relevant targets (identified in the previous step) through molecular docking and molecular dynamics simulations.

For the consensus prediction we used the methodology previously applied in other studies to leverage all potential pertinent algorithms (Beltrán-Noboa et al. 2022; Tejera et al. 2022). Briefly, different target fishing models are used to identify potential targets for each alkaloid. These models include: MolTarPred (Peón et al. 2017), SwissTargetPrediction (Daina et al. 2019), TargetNet Scbdd (Yao et al. 2016), TargetNet Scbdd -Ensemble (Yao et al. 2016), RF QSAR (Lee et al. 2017) and PPB2 (Awale and Reymond 2018). With PPB2 we used the following algorithms: PPB2—Extended Connectivity fingerprint ECfp4 NN, PPB2—Shape and Pharmacophore fingerprint Xfp NN, PPB2—Molecular Quantum Numbers MQN NN, PPB2—Extended Connectivity fingerprint ECfp4 NNNB, PPB2—Shape and Pharmacophore fingerprint Xfp NNNB, PPB2—Molecular Quantum Numbers MQN NNNB, PPB2—Extended Connectivity fingerprint ECfp4 NB and PPB2—Extended Connectivity fingerprint DNN. Each of these models produces a list of potential targets for each alkaloid, based on targets in *Homo sapiens* because it is the only species common to all predictions. To obtain a final score (*FSj)* of all target proteins we integrated individual predictions across all models using the formula for each target "*j*" as:

$$FS_j = \sqrt{\frac{F_j}{M}\frac{1}{M}\sum_{i=1}^{M} S_{i,j}}$$

where *M* is the number of molecules, *Fj* is the number of molecules interacting with the target and *Si,j* is the rank-normalized score of the interaction between the compound *i* and

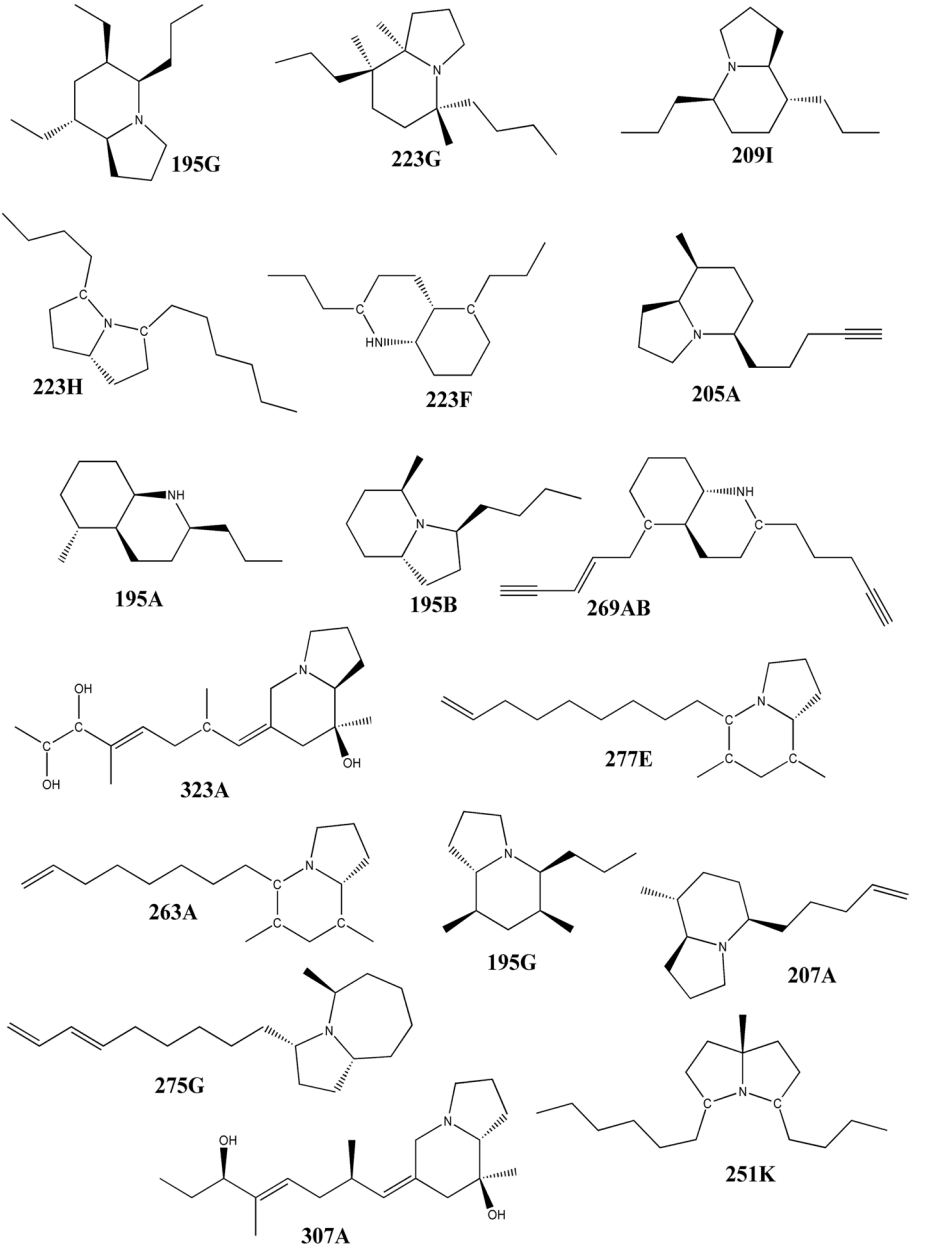

Fig. 1 Seventeen alkaloids were obtained from *O. pumilio* skin, divided in seven classes: Pumiliotoxins 307A and 323A; 3,5-disubstituted indolizidines 195B and G; Lehmizidine 275G; 5,8-disubstituted indolizidine 205A, 207A, 209I and 223 J; 3,5-pyrrolizidine 223H and 251 K; DHQ 195A, 223F and 269AB; and 5,8,7-trisubstituted indolizidine 223A, 263A and 277E

the protein *j*. The variable *FSj* is a measure of the confidence across all methods of the interaction between all alkaloids and the target "*j*". Considering that computational target fishing algorithms are biased in favor of human ligand-receptor interactions, only *H. sapiens* proteins were considered for consensus predictions.

Homologous search of muscarinic receptors

We used human muscarinic receptors identified from the previous section (see also results) as query proteins for the BLAST search in the proteomes of other (*Nanorana parkeri, Xenopus laevis, Xenopus tropicalis, Dendrobates tinctorius* and *Gallus gallus*) representing species with available genomes which help us to model potential interactions such as with conspecifics (preventing autotoxicity) and various predator classes (chemical defenses) with frog alkaloids. Additionally because some natural history observations suggest arachnids and serpents can consume poison frogs (Santos and Cannatella 2011) we carried out a preliminary search including all available sequences in GenBank for snake (*Notechis scutatus*, *Python bivittatus*, *Ophiophagus hannah)* and spider species (*Caerostris darwini, Argiope bruennichi*, *Parasteatoda tepidariorum*). Available sequences unfortunately do not represent exact predators (most do not overlap geographically with *O. pumilio*), however we found muscarinic receptors, orthosteric and allosteric sites were all highly conserved in spiders and snakes, as we observed in chicken, humans and frogs (Table S2). Due to computational limitations further analyses were narrowed excluding arachnids and serpents, though we note these other predator classes should be further explored in future studies. The best hits obtained from BLAST searches were globally aligned using MAFFT 7 (using default options, Katoh and Standley 2013)), and orthosteric and allosteric sites were compared with the sites found in *H. sapiens* (Thal et al. 2016). In order to assess whether these candidate genes are under selection, we used Fast Unconstrained Bayesian AppRoximation (FUBAR), Fixed Effects Likelihood (FEL) and Mixed Effects Model of Evolution (MEME) from the HyPhy 2.5 package (Kosakovsky Pond et al. 2020) through the Datamonkey Web server (Weaver et al. 2018). The selection analyses exclusively utilized cDNA sequences from Humans, *Nanorana parkeri*, *Xenopus laevis*, and *Xenopus tropicalis*. This decision was made due to the uncertain annotation of muscarinic receptor genes in *Gallus gallus* or *Dendrobates tinctorius*.

Modeling frog alkaloid-muscarinic interactions

Receptor and ligand preparation

The structures of the human muscarinic receptors CHRM1 (code 6WJC), CHRM2 (5ZKC), CHRM4 (5DSG) and CHRM5 (6OL9) were retrieved from the Protein Data Bank (PDB) database. Homology models were generated for the human CHRM3 (code 2CSA) receptor and the muscarinic receptors from *N. parkeri*, *X. laevis*, *X. tropicalis*, *D. tinctorius* and *G. gallus* with the SwissModel web server (Biasini et al. 2014). Different templates were considered for building the homology models and the model with the highest QMEAN score was selected. Alkaloids were prepared for modeling by generating one initial three-dimensional conformation for each one of the 12 alkaloids studied with OpenEye's Omega (Omega version 4.1.0.2; QUACPAC version 2.1.1.2; OpenEye Scientific Software, Santa

Fe, NM.) and am1-bcc partial atomic charges were added to these with OpenEye's MolCharge (Hawkins et al. 2010).

Molecular docking against muscarinic acetylcholine receptors

All 17 alkaloids were docked into each muscarinic receptor from all species using the Gold software (Jones et al. 1997). Docking calculations were performed with the compound considered flexible. Thus, the compound's conformational space is explored during docking calculations. The ChemPLP scoring function was used to explore 30 different solutions for each compound-target pair. The search efficiency parameter of Gold was set to 200%. The resulting 30 poses per compound were rescored with Gold's scoring functions ChemScore, ASP and GoldScore. Afterwards, scores of the compound's poses were converted to Z-scores at a compound level, and aggregated as described in previous publications (Lopes et al. 2019; Turkez et al. 2019). Score scaling to Z-scores was performed from the average ($\overline{S}$) and standard deviation (std(S)) of the scoring values for all compound poses according to one scoring function $\mathrm{Zi} = \frac{\mathrm{S}_i - \overline{S}}{(\mathrm{std(S)})}$, where S_i represents the score of pose i according to the scoring function. The final binding mode of each compound to a receptor was selected as that with the highest value of aggregated Z-score.

Docking scores of the predicted binding modes of all compounds (one per compound) to a receptor were again converted to Z-scores considering their scoring values for scaling. The final aggregated scores were used as a ranking criterion to sort the compounds from best (rank 1) to lowest (rank 17) probabilities to bind to a receptor. Finally, the rank of all compounds across all muscarinic receptors of one species were averaged and the chemical with the lowest average ranking was selected as the one with the best profile of binding to all receptors simultaneously.

Molecular dynamics simulations and free energies of binding

All ligand-receptor complexes were prepared for molecular dynamics (MD) simulations with the CHARMM-GUI web server (Jo et al. 2008; Wu et al. 2014; Lee et al. 2016). Complexes were embedded in a lipid bilayer containing, on each side, 60 POPC, 60 POPE and 30 cholesterol molecules. The systems containing the receptor, the ligand and the lipid bilayer were solvated and neutralized with OPC water molecules and 0.15 M of KCl, respectively. The Amber ff19sb and gaff2 force fields were selected to parametrize proteins and ligands, respectively. All MD simulations were performed with Amber 20 (Case et al. 2021). The energy minimization, heating and equilibration steps were conducted using the configuration files provided by the CHARMM-GUI server. The equilibrated systems were used as input to five short (2 ns) production runs, each one initialized with different random velocities. Production runs included no constraints on the dynamics of the system.

The estimation of the free energies of binding was carried out with the MM-PBSA method as implemented in Amber 20. A total of 100 MD snapshots were considered for calculations and these were evenly selected from all the five short molecular dynamic simulations. A heterogeneous dielectric implicit membrane model (memopt = 3) was selected for MM-PBSA calculations and the solute dielectric constant was set to 2. The membrane thickness and its center were defined on a system basis as the average distance between the N31 atoms in the membrane and the average z-coordinate of the same set of atoms, respectively, along the total 10 ns simulation of each system.

Results

Likely target proteins and their affinity for poison frog alkaloids

From consensus analysis, several human proteins were identified as likely targets of the defensive alkaloids of *O. pumilio*, the top 20 candidates are presented in Table 1. Muscarinic receptors were among the most likely of the predicted targets of frog alkaloids, and the most highly represented group. We focused on this diverse group of receptors as a preliminary pass at identifying novel targets to better understand the defensive function of alkaloids. Due to computational demands, we chose to explore a single group rather than taking a broader approach which would be less likely to afford higher precision in resolving specific frog/receptor interactions. However, we note there are numerous other potential targets we recovered that are worth assessing in future analyses.

Homology in muscarinic receptors

Our initial predictions are based on *H. sapiens* muscarinic receptors, therefore, we carried out protein sequence alignments to assess potential homology in other biologically relevant species. Muscarinic receptors are G-coupled receptors which serve highly diverse biologically essential roles in a variety of species (Eglen 2005). Although we attempted preliminary analyses on three poison frog genomes (*O. pumilio, Ranitomeya imitator and D. tinctorius*) to infer whether they may have any mechanisms for avoiding autotoxicity, our final analyses only report *D. tinctorius* (Comeault et al., unpublished data) due concerns in the quality or completeness of other genomes. As a comparison, of the additional frog genomes available we chose three additional frog species to compare with the human CHRMs through BLAST searches (*N. parkeri, X. tropicalis* and *X. laevis*), as well as a bird (*G. gallus*) representative of avian frog predators (Comeault and Noonan 2011).

The detected homologues were aligned and compared the allosteric, orthosteric and hybrid (sites that work either as allosteric or orthosteric) sites of muscarinic acetylcholine receptors CHRMs 1 to 5 (Figures S1-S5), as described in (Thal et al. 2016). We observed that orthosteric and hybrid sites are highly conserved (supplementary Tables S2-S6, and supplementary Figures S1-S5). However, allosteric sites show several amino acid changes which were analyzed downstream with molecular dynamics simulations.

For the most part CHRMs sequences represent neutral sites (based on consensus between FEL, FUBAR and MEME models) where allosteric, orthosteric and hybrid sites show strong evidence of purifying selection (see supplementary Tables S2-S6). Sites under positive selection were rare, and inconsistently identified (e.g., varied between selection detected vs. absent) between FEL, FUBAR and MEME predictions. Together, our results demonstrate that CHRMs are highly conserved through phylogenetically distant species (e.g., mammals, birds and amphibians) (Table 2).

Molecular docking and molecular dynamic simulations

Homology models were generated for the muscarinic receptors from non-chemically defended frogs *N. parkeri*, *X. laevis*, *X. tropicalis*, and chicken (*G. gallus*), as well as for the human CHRM3 receptor (described in the methods). The models were visually

Table 1 Probable human target for frog alkaloids obtained from consensus analysis. For each human protein, the final score, FPS_j, was calculated to indicate how likely that protein interacts with all 17 alkaloids. Gene symbol and protein name are also included for identification

Proteins name	FPS	Gene symbol
Muscarinic acetylcholine receptor M4	0.828	CHRM4
Muscarinic acetylcholine receptor M5	0.768	CHRM5
Alpha-2B adrenergic receptor (Alpha-2 adrenergic receptor subtype C2) (Alpha-2B adrenoreceptor) (Alpha-2B adrenoceptor) (Alpha-2BAR)	0.752	ADRA2B
5-hydroxytryptamine receptor 1E (5-HT-1E) (5-HT1E) (S31) (Serotonin receptor 1E)	0.748	HTR1E
D(1B) dopamine receptor (D(5) dopamine receptor) (D1beta dopamine receptor) (Dopamine D5 receptor)	0.733	DRD5
Neuronal acetylcholine receptor subunit alpha-4	0.727	CHRNA4
Muscarinic acetylcholine receptor M1	0.725	CHRM1
Sphingosine 1-phosphate receptor 2 (S1P receptor 2) (S1P2) (Endothelial differentiation G-protein coupled receptor 5) (Sphingosine 1-phosphate receptor Edg-5) (S1P receptor Edg-5)	0.724	S1PR2
DNA dC- > dU-editing enzyme APOBEC-3A (A3A) (EC 3.5.4.38) (Phorbolin-1)	0.721	APOBEC3A
D(2) dopamine receptor (Dopamine D2 receptor)	0.721	DRD2
Alpha-2C adrenergic receptor (Alpha-2 adrenergic receptor subtype C4) (Alpha-2C adrenoreceptor) (Alpha-2C adrenoceptor) (Alpha-2CAR)	0.720	ADRA2C
Nitric oxide synthase, brain (EC 1.14.13.39) (Constitutive NOS) (NC-NOS) (NOS type I) (Neuronal NOS) (N-NOS) (nNOS) (Peptidyl-cysteine S-nitrosylase NOS1) (bNOS)	0.718	NOS1
5-hydroxytryptamine receptor 5A (5-HT-5) (5-HT-5A) (5-HT5A) (Serotonin receptor 5A)	0.716	HTR5A
Nitric oxide synthase, inducible (EC 1.14.13.39) (Hepatocyte NOS) (HEP-NOS) (Inducible NO synthase) (Inducible NOS) (iNOS) (NOS type II) (Peptidyl-cysteine S-nitrosylase NOS2)	0.705	NOS2
Nitric oxide synthase, endothelial (EC 1.14.13.39) (Constitutive NOS) (cNOS) (EC-NOS) (Endothelial NOS) (eNOS) (NOS type III) (NOSIII)	0.705	NOS3
Sigma non-opioid intracellular receptor 1 (Aging-associated gene 8 protein) (SR31747-binding protein) (SR-BP) (Sigma 1-type opioid receptor) (SIG-1R) (Sigma1-receptor) (Sigma1R) (hSigmaR1)	0.703	SIGMAR1
Muscarinic acetylcholine receptor M2	0.700	CHRM2
Neuronal acetylcholine receptor subunit alpha-7	0.700	CHRNA7
Monoglyceride lipase (MGL) (EC 3.1.1.23) (HU-K5) (Lysophospholipase homolog) (Lysophospholipase-like) (Monoacylglycerol lipase) (MAGL)	0.680	MGLL

Table 1 (continued)

Proteins name	FPS	Gene symbol
Polyphenol oxidase 2 (PPO2) (Phenolase 2) (EC 1.14.18.1) (Cresolase 2) (Tyrosinase 2)	0.679	PPO2

Table 2 Homologous human muscarinic receptors in frogs *X. laevis, X. tropicalis, N. parkeri, Dendrobates tinctorious* (see Supplementary tables S1-S5) and chickens (*G. gallus*) obtained by BLAST searches

Muscarinic receptor	Species	Accession	Query cover (%)	E. Value	Identity (%)
CHRM1	*X. laevis*	XP_018113038.1	99	0.0	56.78
	X. tropicalis	XP_004913717.1	95	0.0	57.17
	N. parkeri	XP_018409795.1	99	0.0	57.11
	G. gallus	NP_001351587.1	91	7,00E-167	51.52
	D. tinctorius[*]		100		81
CHRM2	*X. laevis*	XP_018110268.1	100	0.0	80.30
	X. tropicalis	XP_002942846.2	100	0.0	81.36
	N. parkeri	XP_018414063.1	100	0.0	80.93
	G. gallus	NP_001025936.1	100	0.0	85.93
	D. tinctorius[*]		100		90.30
CHRM3	*X. laevis*	XP_018118616.1	99	0.0	62.90
	X. tropicalis	XP_002935876.2	100	0.0	63.29
	G. gallus	NP_990730.1	99	0.0	79.53
CHRM4	*X. laevis*	XP_018115713.1	100	0.0	72.76
	X. tropicalis	NP_001106514.1	96	0.0	74.15
	N. parkeri	XP_018424401.1	100	0.0	71.14
	G. gallus	XP_015142523.1	100	0.0	74.44
	D. tinctorius[*]		99		83.5
CHRM5	*X. laevis*	XP_018087696.1	96	0.0	72.34
	X. tropicalis	XP_017952476.1	98	0.0	70.57
	N. parkeri	XP_018409705.1	98	0.0	72.32
	G. gallus	AAF19027.1	100	0.0	76.59
	D. tinctorius[*]		100		90.1

*Homologues in *D. tinctorius* were detected by tblastn in the assembly genome of the species, using the *X. laevis* protein as a query. For all these species, we found high values of identity (90.3–83.5%) and query coverage (100–99%) (Table 2), showing remarkable levels of conservation

inspected, and a few (6 models among 3 species) were discarded due to their low quality, e.g., insertions were present in the transmembrane region, or the cytoplasmic domain was too distorted. The list of remaining muscarinic receptors for modeling studies is presented in Table 3.

Lehmizidine 275G was found to have the strongest binding to all muscarinic receptors, across all target species (except *X. tropicalis* when it was the second best). For this reason, we selected Lehmizidine 275G as the representative alkaloid for molecular dynamics simulations to predict the free energies of binding for all the muscarinic receptors. Detailed results of the molecular docking calculations are provided in the supplementary materials (Table S7).

Calculated free energies of binding of Lehmizidine 275G to all muscarinic receptors (Fig. 2) and the predicted energy values as well as their components are given in the supplementary materials (Table S8, Table S9). For replicability and comparisons, the same modeling protocol was applied to the X-ray structure of the human CHRM2 receptor in complex with N-methyl scopolamine (PDB code 5ZKC) which was used as a reference of known binding capabilities to the human CHRM2 receptor. We find the same binding

Table 3 List of the muscarinic receptors selected for modeling studies

Species	Receptor	Uniprot ID	ID[(a)]
Homo sapiens	CHRM1	P11229	hsCHRM1
	CHRM2	P08172	hsCHRM2
	CHRM3	P20309	hsCHRM3
	CHRM4	P08173	hsCHRM4
	CHRM5	P08912	hsCHRM5
Gallus gallus	CHRM2	P30372	ggCHRM2
	CHRM4	P17200	ggCHRM4
	CHRM5	F1P0I0	ggCHRM5
Nanorana parkeri	CHRM1	XP_018409795[b]	npCHRM1
	CHRM2	XP_018414063[b]	npCHRM2
	CHRM4	XP_018424401[b]	npCHRM4
	CHRM5	XP_018409705[b]	npCHRM5
Xenopus laevis	CHRM1	A0A1L8GJI8	xlCHRM1
	CHRM2	A0A1L8GQL0	xlCHRM2
	CHRM4	A0A1L8GDU2	xlCHRM4
Xenopus tropicalis	CHRM1	A0A6I8SRX2	xlCHRM1
	CHRM2	F7A3M1	xlCHRM2
	CHRM4	A0A803J2D1	xlCHRM4

[(a)]ID used for each receptor along the manuscript

[(b)]ID from NCBI

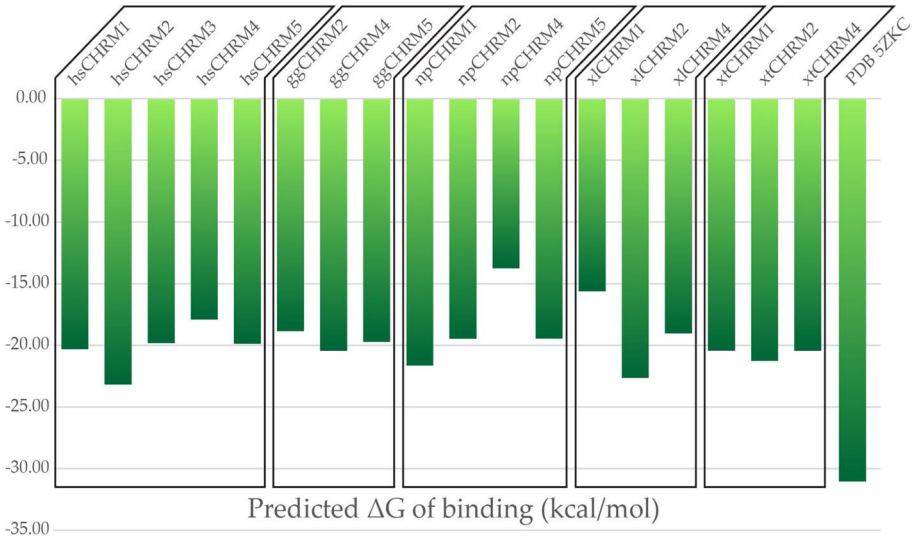

Fig. 2 Free energies of binding of Lehmizidine 275G to all CHRMs from all the species used for the analysis. The vertical axes correspond to the predicted free energies of binding. Lower binding energies are indicative if higher complex stability. Receptors from different species are labelled according to Table 3 and include *Homo sapiens* (hs), *Gallus gallus* (gg), *Nanorana parkeri* (np), *Xenopus laevis* (xl) and *Xenopus tropicalis* (xt). The PDB code 5ZKC corresponds to the experimentally determined complex of the human CHRM2 receptor with N-methyl scopolamine

profile for the predicted free energies of binding Lehmizidine 275G to all muscarinic receptors, with similar ranges of binding energy (from -13.76 kcal/mol to −23.17 kcal/mol) as well. The average value of these energies is -19.85 kcal/mol, which represents 64% of the predicted free energy of binding N-methyl scopolamine to the human CHRM2 receptor.

Modeling experiments showed similar free energies of binding of Lehmizidine 275G to all evaluated muscarinic receptors (Fig. 2). Furthermore, these binding energies are similar to that obtained for the experimentally determined complex of N-methyl scopolamine with the human CHRM2 receptor (see Fig. 3). Similar binding energies are a consequence of completely conserved binding sites across all muscarinic receptors, and the similarity between the binding regions and similar binding orientations of N-methyl scopolamine and Lehmizidine 275G to the human CHRM2 receptor, Fig. 3). This figure shows the high overlap of the binding regions for the two compounds. There are 11 residues interacting with the two ligands, two interactions are exclusive for N-methyl scopolamine and one exclusive interaction is predicted for Lehmizidine 275G. In addition, all the amino acids interacting with the alkaloid in its predicted complex with hsCHRM2 are conserved among all of the muscarinic receptors studied. The lack of the hydrogen bonds of N-methyl scopolamine to N404 in the predicted complex with Lehmizidine 275G could explain the observed differences in free energies of binding for these molecules to muscarinic receptors.

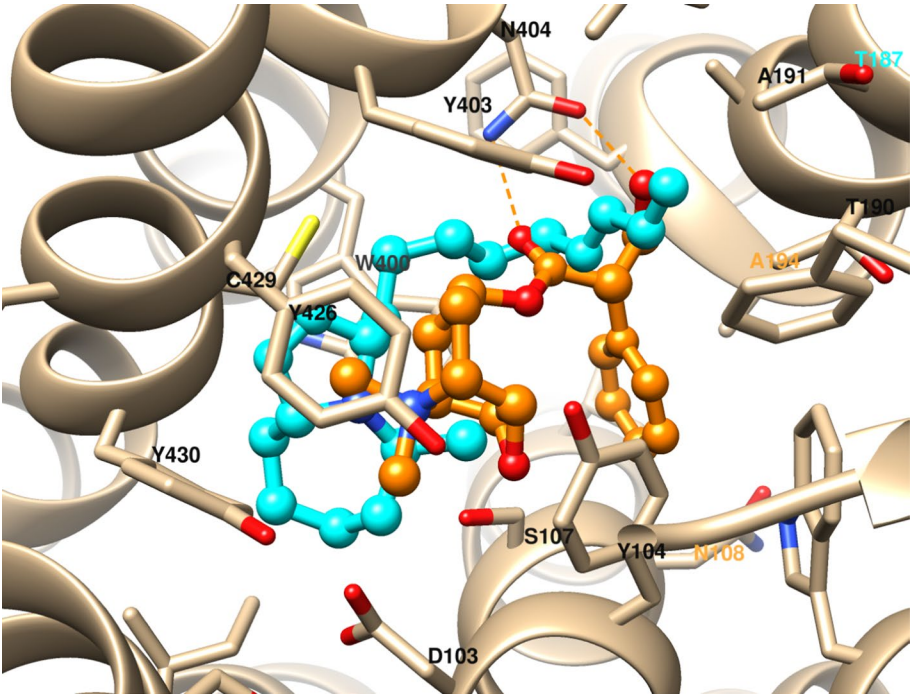

Fig. 3 Predicted binding modes of N-methyl scopolamine (a molecule known to bind to muscarinic receptors, orange) and Lehmizidine 275G (predicted interaction, cyan) to the human CHRM2 receptor. Residues interacting with both ligands are labeled black, exclusive interactions with N-methyl scopolamine are orange and interactions only observed in the complex with Lehmizidine 275G are labeled cyan. The hydrogen bonds of N-methyl scopolamine to N404 are represented by dashed orange lines. The structures used for depiction were the centroids of the most populated clusters obtained from clustering the MD snapshots employed for MM-PBSA calculations

Summed, these results led us to hypothesize that Lehmizidine 275G and N-methyl scopolamine could have a similar effect of binding on muscarinic receptors across all receptor isoforms. That could explain, at least in part, the defensive properties of the alkaloids present among these *O. pumilio* populations due to the precise similarity of binding orientation and intermolecular contacts between the ligands and the receptors. Interestingly the binding site of Lehmizidine 275G to muscarinic receptors is conserved even in a poison frog species, suggesting there is no mechanism, at least in muscarinic receptors, for avoiding potential autotoxicity effects. However, confirmation of this would require additional experimentation.

Discussion

Variation in chemical defenses is the lesser understood component of aposematic signal divergence (Speed et al. 2012), particularly across geographic scales in poison frogs. While we continue to progress in our ability to analyze saliency in visual signals across diverse predator types (Crothers and Cummings 2013; Barnett et al. 2017; Yeager et al. 2022), similar advances will be essential to improve our understanding of, and ability to compare between the defensive functions of different secondary metabolites. This is particularly the case in aposematic species with extensive defensive portfolios. Our modeling has revealed numerous potential targets for common alkaloids found in *O. pumilio*, where we specifically identified strong support for classes of muscarinic acetylcholine receptors as targets for binding with frog alkaloids. Interestingly, we found these receptors are highly conserved between phylogenetically divergent species, due to strong purifying selection present on these receptors.

While pumiliotoxins (some of which are included in our analyses) have been shown to modulate sodium and calcium transport during muscular contractions, to date, no information has been described regarding possible effects on muscarinic receptors (Daly et al. 1999). Muscarinic receptors consist of different subtypes (for example CHRM1-CHRM5 in mammals) where they play a role in the parasympathetic nervous system (Pedersen and Bergqvist 2018; Kudlak and Tadi 2021). In humans the influence of each muscarinic receptor subtype can be organ specific. For example, CHRM1 is expressed in the brain and it is related to cognitive functions, while CHRM2 is associated with changes in heart rate. Interestingly, CHRM1and CHRM3 are known to be expressed in the salivary glands of humans and rodents, and they are related to gland secretion (specially CHRM3). These receptors could therefore be important in binding with alkaloids early in predator education as they sample prey, or as prey are consumed by mammalian predators (Abrams et al. 2006). In birds, studies also found expression of subtypes of muscarinic receptors in the brains, possibly related to song production in passerines (Ball et al. 1990; Jaffe and Brainard 2020). It is plausible that for avian predators, frog chemical defenses could possibly influence some sort of behavioral processes related to avian brain function if alkaloids could pass the blood–brain barrier (parallels described in Ejsmond and Provenza 2018), however these specific mechanisms of how alkaloids contribute to defensive functions are as of yet unclear.

Molecular docking experiments between all alkaloids and muscarinic receptors indicated that Lehmizidine 275G has a high potential for binding to muscarinic receptors among the evaluated alkaloids. The predicted Lehmizidine 275G-muscarinic receptor complexes were subject to molecular dynamics simulations that yield free binding energies favorable for the

formation of these complexes. This suggests that Lehmizidine 275G highly likely binds to all muscarinic receptor types. We found muscarinic receptors are highly conserved across species, and molecular docking experiments suggest that binding between diverse sampled alkaloids and receptors is similarly likely. Yet our strongest specific support comes specifically from molecular dynamics results for Lehmizidine 275G binding equally well across muscarinic receptor subtypes in diverse species. Based on this evidence we predict it is likely there could be parity in binding, indicating similar functions of chemical defenses for Lehmizidine 275G with muscarinic receptors across diverse potential predator species. We cannot conclusively state this without substantial further confirmations of molecular dynamics models for all receptors and alkaloids, which is beyond our computational capacity, although our results strongly suggest this is could the case. We do note that conclusions derived from modeling approaches (such as molecular docking) carry a risk of inaccuracy, which we have mitigated using confirmational molecular dynamics simulations, which we additionally benchmarked with a known experimental complex of a muscarinic receptor with a ligand (scopolamine). Further studies are needed to investigate whether the trends predict are found more widely across other common defensive alkaloids, and other alkaloid binding sites.

While we are not able to directly describe the defensive effects of frog alkaloids on muscarinic receptors, our results suggest several distinct findings. First, all alkaloids are predicted to be highly likely to bind to numerous subtypes of muscarinic receptors, across various predator classes. Second, in the genomes surveyed, we find muscarinic receptors are under strong purifying selection indicating that similar binding potentials are likely, a finding further supported by modeling experiments which led us to hypothesize that these alkaloids defensive functions are likely similar. We also note that the binding site of Lehmizidine 275G to muscarinic receptors is predicted to be conserved even in a poison frog species, suggesting there is no mechanism, at least in muscarinic receptors, for avoiding potential autotoxicity effects.

Because defensive functions are predicted to be conserved, shifts between the alkaloid types we surveyed, which could include those due to changes in invertebrate prey availability may not diminish the function of chemical defense. Therefore predation risk should remain reduced by aposematic signals among diverse classes of potential predators. While far from conclusive, our results suggest there may be potential mechanisms by which common alkaloids found across phenotypically divergent populations of *O. pumilio* could function to promote avoidance by diverse would-be predators. Additional studies are needed to more clearly disentangle specific effects between alkaloid types and muscarinic classes, in addition to the numerous other potential binding targets our analyses have recovered.

Without an alkaloid-specific understanding of functions it has remained elusive if, and how, variance in chemical defense profiles may be meaningful (Lawrence et al. 2019, 2023). In aposematic species such as *O. pumilio,* important predator/prey dynamics are influenced by honest aposematic signals (Maan and Cummings 2012), where predators learn to associate signals with a known level of chemical defense (Summers et al. 2015). It has previously been proposed that different co-occurring ant species may be redundant dietary sources of specific alkaloids for *O. pumilio* (Prates et al. 2019). Functional redundancy, or any significant overlap in the defensive functions of common alkaloids across phenotypically distinct populations, would further ensure defenses vary minimally. This should help stabilize defenses for instances when alkaloid-rich prey are unstable, or vary temporally or geographically (Saporito et al. 2007). This would specifically insulate frog populations from potential maladaptive situations or mismatches between visual elements of aposematic signals and the chemical defenses they advertise.

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Author contributions JY, YP-C, VA-J and ET designed the experiment, PAV-C, YP-C, VA-J and ET conducted modeling with contributions from JY, JY wrote the manuscript with contributions from YP-C, VA-J and ET.

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Data availability All data and explanation are present in the supplementary materials.

Code availability All data and explanation are present in the supplementary materials.

Declarations

Conflict of interest The authors declare no competing interests influenced the study.

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