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Actions of piperidine alkaloid teratogens at fetal nicotinic acetylcholine receptors

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ABSTRACT

Teratogenic alkaloids are found in many species of plants including *Conium maculatum L.*, *Nicotiana glauca*, *Nicotiana tabaccum*, and multiple *Lupinus* spp. Fetal musculoskeletal defects produced by alkaloids from these plants include arthrogyropisis, scoliosis, torticollis, kyposis, lordosis, and cleft palate. A pharmacodynamic comparison of the alkaloids ammodendrine, anabasine, anabaseine, anagyrine, and coniine in SH-SY5Y cells and TE-671 cells was made. These alkaloids and their enantiomers were more effective in depolarizing TE-671 cells which express the human fetal-muscle type nicotinic acetylcholine receptor (nAChR) relative to SH-SY5Y cells which predominately express autonomic nAChRs. The rank order of potency in TE-671 cells was: anabaseine > (+)-anabasine > (−)-anabasine > (±)-anabasine > anagyrine > (−)-coniine > (±)-coniine > (+)-coniine > (±)-ammodendrine > (+)-ammodendrine. The rank order potency in SH-SY5Y cells was: anabaseine > (+)-anabasine > (−)-coniine > (+)-coniine > (+)-ammodendrine > anagyrine > (−)-anabasine > (±)-coniine > (±)-anabasine > (−)-ammodendrine. The actions of these alkaloids at nAChRs in both cell lines could be distinguished by their maximum effects in depolarizing cell membrane potential. The teratogenic action of these compounds may be related to their ability to activate and subsequently desensitize nAChRs.

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1. Introduction

Teratogenic alkaloids (Fig. 1) are found in many species of plants including *Conium maculatum* (poison hemlock), *Nicotiana glauca* (wild tree tobacco), *Nicotiana tabaccum* (tobacco) and multiple *Lupinus* spp. (lupine). Fetal defects caused by alkaloids from these plants are a multiplicity of contractive skeletal defects including arthrogyropisis, scoliosis, torticollis, kyposis, lordosis, and associated skeletal defects [29,31]. Cleft palate is also associated with these skeletal malformations. The period of gestation when the fetus is susceptible to these plant teratogens varies with livestock species but in general, susceptible periods are 30–45 days for cleft palate, and 50–100 days for forelimb spine and neck defects. Specific insult periods and species differences have been recently reviewed [27].

The mechanism behind these fetal defects is thought to be the inhibition of fetal movement [26], due to stimulation followed by desensitization of skeletal muscle-type nicotinic acetylcholine receptors (nAChR) [21]. When pregnant Spanish-type goats are dosed with *N. glauca*, which contains high concentrations of anabasine, there is an initial period of voluntary muscular contractions followed by severe muscle weakness or paralysis for periods up to 60 min in duration [28]. These effects of *N. glauca* on fetal movements were observed long after the dam had recovered from intoxication. From this observation it was hypothesized that inhibition of fetal movement is due to the desensitization of nAChR, and that fetal deformities occur if the piperidine alkaloids meet certain structural requirements [27].

Teratogenic piperidine alkaloids are also produced by *Conium* and *Nicotiana* species. *C. maculatum* is known to produce at least eight piperidine alkaloids [12,23,26]. In the mature plant and seed, coniine, a nAChR agonist, predominates and is both acutely toxic as well as teratogenic and is found as a mixture of the two enantiomers [4,9,19,24,30]. *N. glauca* produces the piperidine alkaloid anabasine, a nAChR agonist, that is present in the plant as a racemate [4,5,11]. The chemically related alkaloid anabaseine, though not reported to occur in tobacco, is found in certain marine worms and in ants [14,33], and is a potent agonist at neuromuscular nAChRs [15].

Teratogenic members of the genus *Lupinus* contain the quinolizidine alkaloid anagyrine and/or, the piperidine alkaloid ammodendrine, or *N*-methyl ammodendrine [27]. The quinolizidine alkaloid anagyrine is teratogenic in cattle but not in sheep, goats, or hamsters [10,29]. It has been hypothesized that anagyrine may be metabolized in the bovine rumen to a piperidine alkaloid, which is then absorbed as a complex piperidine with teratogenic activity. However, data from kinetic experiments in cattle, sheep, and goats suggest that it is not ruminally or hepatically biotransformed [7,10,13]. The potency of anagyrine relative to the piperidine alkaloids in cell based assays, or

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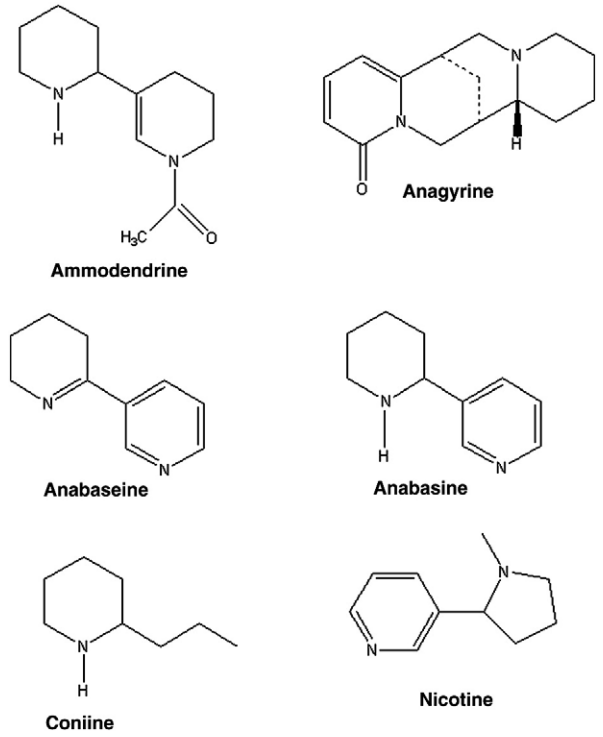

Fig. 1. Chemical structures of piperidine and quinolizidine alkaloid teratogens. The structure of the pyridine alkaloid nicotine, is included for comparison.

toxicity in a mouse-based bioassay, have yet to be determined. Ammodendrine is a piperidine alkaloid found in lupine species, it acts as a nAChR agonist, and has been found in plants as a mixture of enantiomers [4,20]. Although the LD_{50} of ammodendrine has been determined in a mouse-based bioassay [20], there have been no studies of the potency and efficacy of ammodendrine in cell-based assays.

Investigations of the actions of several of these alkaloids have provided some insight regarding the chemical basis for these fetal malformations and determination of a teratogenic piperidine alkaloid activity profile (TAP) which is based on piperidine alkaloid structure, relative teratogenicity in ruminants, and relative lethality in a mouse model. Researchers [9] first proposed that piperidine alkaloids with a carbon chain at least three carbons or larger and attached to the carbon alpha to the piperidine nitrogen have teratogenic activity. Further structural features that affect teratogenic potency include a double bond between the nitrogen and alpha side chain (i.e., γ-coniciene) or conversely, a methyl group attached to the nitrogen reduces potency [28]. In addition to differences in TAP, some of these alkaloids possess chiral centers and the enantiomers possess different biological activities [19–21].

In pregnant goats dosed with plants which contain teratogenic piperidine alkaloids, there are clinical signs consistent with nAChR stimulation followed by inhibition. These clinical signs are usually short lived in the mother, but persist in the developing fetus. This may be due to differences in the subunit composition of nAChR expressed by the developing fetus. In this study we compared the pharmacodynamic actions of anabasine, anabaseine, coniine, ammodendrine, and anagyrine on nAChRs expressed in TE-671 cells and SH-SY5Y cells. Both of these cell lines have been well characterized in assays of nAChR function and both have fetal characteristics [6,8]. TE-671 cells express fetal human muscle-type nAChR ($\alpha1_2\beta1\gamma\delta$). SH-SY5Y cells on the other hand express several nAChR subunits, including α3, α5, α7, β2, and β4, and under the conditions of our functional assays, the autonomic type nAChRs containing α3 and β4 subunits are dominant [8,22]. In this study, we had two specific aims: (1) compare the actions of five teratogenic plant alkaloids at human fetal muscle-type nAChR and human autonomic nAChR, and (2) identify a cell line which can serve as a possible *in vitro* model for teratogenic alkaloids.

2. Materials and methods

2.1. Materials

Fetal bovine serum was obtained from Hyclone, Inc. (Logan, UT). Penicillin/streptomycin was obtained from Invitrogen (Carlsbad, CA). Dulbecco's modified Eagle's medium was from the American Type Culture Collection (Manassas, VA), and the fluorescence dye kits were purchased from Molecular Devices (Sunnyvale, CA).

2.2. Acute toxicity determinations

Known amounts of anagyrine were dissolved in physiological buffered physiological saline solution. The solutions were stored in sterile injection vials for toxicity testing. Swiss-Webster male mice, 15 to 20 g (Simonsen Labs, Gilroy, CA) were weighed after a 12 h fast and dosed intravenously via the tail vein. Prior to injection, the mice were maintained under a heat lamp for 15 min to produce vasorelaxation. The tail surface was cleaned with 70% ethanol and i.v. injections were accomplished with a tuberculin syringe equipped with a 1.27-cm-long 27-gauge needle. The volume injected (0.05–0.2 mL) varied depending on the alkaloid dose delivered. Time of injection, clinical effects, and time of death were noted and recorded. Mice were closely observed for 1 h after injection. The LD_{50} for anagyrine was determined by a modified up-and-down method [3] and was calculated using the PROC PROBIT procedures of SAS (SAS Institute Inc., Cary, NC) on a logistic distribution of the survival data.

2.3. Chemistry

The separation and analysis of plant alkaloids was done as previously described [18–21]. Anabaseine was synthesized as previously described [2].

2.4. Cell-based assays

The human rhabdomyosarcoma cell line TE-671 and the human neuroblastoma cell line SH-SY5Y were obtained from ATCC (Manassas, VA, USA). Membrane depolarization responses from the addition of nAChR agonist toxins were measured by changes in fluorescence of a membrane potential-sensitive dye as previously described [19] with the following modifications. The membrane potential dye solution was prepared by dissolving one vial of the Molecular Devices dye (Catalog number R8042) into 22 mL Hanks' balanced salt solution (HBSS) supplemented with 20 mM Hepes (pH 7.4). Ninety-six well black walled cell culture plates were equilibrated to room temperature for 10 min, then the medium was aspirated and replaced with 100 μL of the membrane potential dye solution in each well. Dye loading times at room temperature were optimized for each cell type according to manufacturer recommendations [25]. TE-671 cells were loaded with dye for 30 min whereas SH-SY5Y cells were loaded with dye for 60 min. Serial dilutions of a compound for concentration-response analysis were prepared in 96- well V-bottom plates by addition of the required volume of a methanolic stock solution. After evaporation of the methanol, the compound in each well was redissolved in membrane potential dye solution.

Fluid (agonist or KCl) additions and membrane potential measurements were performed using a Flexstation II (Molecular Devices Corporation, Sunnyvale, CA, USA). Readings were taken every 1.12 s for 255 s, for a total of 228 readings per well. The first 17 s were used as a basal reading. At 18 s, 50 μL of a test compound was added to assess agonist activity. At 180 s, 25 μL of KCl in saline was added to attain a final