Diabetogenic Effect of a Series of Tricyclic Delta Opioid Agonists Structurally Related to Cyproheptadine


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The unexpected observation of a hyperglycemic effect of some tricycle-based delta opioid receptor (DOR) agonists led to a series of studies to better understand the finding. Single administration of two novel tricyclic DOR agonists dose dependently elevated rat plasma glucose levels; 4-week toxicology studies confirmed the hyperglycemic finding and further revealed pancreatic β-cell hypertrophy, including vacuole formation, as well as bone dysplasia and Harderian gland degeneration with regeneration. Similar diabetogenic effects were observed in dog. A review of the literature on the antiserotonergic and antihistaminergic drug cyproheptadine (CPH) and its metabolites revealed shared structural features as well as similar hyperglycemic effects to the present series of DOR agonists.

To further evaluate these effects, we established an assay measuring pancreatic β-cell–derived RINm5F cell line, extensively used to study CPH and its metabolites. Like CPH, the initial DOR agonists studied reduced RINm5F cell insulin levels in a concentration-dependent manner. Importantly, compound DOR potency did not correlate with the insulin-reducing potency. Furthermore, the RINm5F cell insulin results correlated with the diabetogenic effect of the compounds in a 5-day mouse study. The RINm5F cell insulin assay enabled the identification of aryl-aryl-amine DOR agonists that lacked an insulin-reducing effect and did not elevate blood glucose in repeated dosing studies conducted over a suprapharmacologic dose range. Thus, not only did the RINm5F cell assay open a path for the further discovery of DOR agonists lacking diabetogenic potential but also it established a reliable, economical, and high-throughput screen for such potential, regardless of chemotype or target pharmacology. The present findings also suggest a mechanistic link between the toxicity observed here and that underlying Wolcott-Rallison Syndrome.

Key Words: pancreatic β-cell toxicity; cyproheptadine; diabetogenic effect; bone dysplasia; PERK; eIF2a; Wolcott-Rallison syndrome.

The antiserotonergic and antihistaminergic drug cyproheptadine (CPH), a tricyclic compound, produces diabetogenic effects in rats and mice, which have been extensively characterized (for a review, see Fischer, 1997). CPH and its several metabolites (e.g., see Fig. 1), including desmethyl CPH epoxide (DMCPH epoxide), desmethyl CPH (DMCPH), and CPH epoxide, inhibit insulin synthesis as well as glucose-stimulated insulin release, albeit to different extents. Thus, CPH is more potent than its metabolites in inhibiting glucose-stimulated insulin release, whereas the metabolites are more potent than CPH in inhibiting insulin synthesis, with a rank order potency of DMCPH epoxide > DMCPH > CPH epoxide > CPH (Chow et al., 1989). CPH is diabetogenic upon repeated administration to rodents, resulting in a reversible change in the function of pancreatic β-cells (Fischer, 1997; Kikkawa et al., 1981). For example, repeated CPH administration in rats induces changes in the endoplasmic reticulum of pancreatic β-cells, namely swelling and vacuole formation, as well as a loss of insulin secretory granules. These changes can be recapitulated in cultured rat pancreatic islets or rat-derived insulin-producing cell lines, such as RINm5F cells.

Species differences in the metabolism of CPH contribute to variations in the pancreatic toxicity of the compound. For example, rats readily form the DMCPH epoxide metabolite, whereas this potent insulin synthesis inhibitor could not be detected in human urine following CPH administration (Hintze et al., 1975). It is not known whether species differences in metabolism or other factors underlie the lack of pancreatic β-cell vacuole formation following CPH administration to mice, rabbits, or hamsters (Wold et al., 1971).

Early investigations revealed that the antihistaminergic or antiserotonergic action of a compound is not required for induction of the diabetogenic effect. For example, although the CPH metabolite DMCPH is virtually devoid of the therapeutic effect of CPH (Engelhardt et al., 1965), it is as active as CPH in inhibiting insulin synthesis (Fischer, 1997). Furthermore, information on the structural requisites for the
The diabetogenic effect of compounds related to CPH was obtained with positional diphenylmethylpiperidine (DPMP) isomers (Fig. 1). Thus, 4-DPMP produces hyperglycemia, whereas 2-DPMP does not (Hintze et al., 1977). The lack of the tricyclic ring in DPMP also indicates that this moiety is not a requirement for the activity.

A drug discovery effort targeting the delta opioid receptor (DOR) led to a series of tricyclic tropanylidine compounds with potent DOR agonist properties. The structure and pharmacology of the delta opioid agonist JNJ-20788560 (JNJ-A) has previously been described (Codd et al., 2009). Structurally, the compound contains a tricycle, a moiety featured in many marketed drugs, including members of the antidepressant and antihistaminergic therapeutic families. Briefly, JNJ-A exhibits a binding affinity at DOR of 2nM and a half maximal effective concentration (EC50) value in a DOR GTPγS functional assay of 5.6nM. Administered orally to rats, it reduces inflammatory hyperalgesia in the complete Freund’s adjuvant radiant heat model of inflammatory hyperalgesia, with an ED50 value of 13.5 mg/kg.

Because early rat toxicology studies revealed that JNJ-A and some related compounds elevated blood glucose levels and induced vacuoles in pancreatic β-cells, an effort to understand the structural and pharmacological basis of this toxicity was undertaken. These studies expanded the structure-activity relationship established for CPH- and DPMP-related hyperglycemic activity, extended the predictiveness of the RINm5F cell assay to the chemotypes studied here and led to the identification of structurally distinct DOR agonists devoid of diabetogenic effects. This work also suggests a mechanistic link between the present findings and those characteristic of Wolcott-Rallison Syndrome (WRS), potentially involving aberrant PERK signaling.

**MATERIALS AND METHODS**

**Materials**

**Chemicals.** DMCPH epoxide as well as Johnson & Johnson (J&J) proprietary compounds (Fig. 2) were synthesized in J&J laboratories. The sources of other compounds and materials are given in each procedure.

**Animals.** All experimental procedures were approved by the J&J Institutional Animal Care and Use Committee and were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of the National Research Council and The International Association for the Study of Pain. Details regarding species and strain used are given with each separate procedure.

**Rat and Dog Toxicology Studies**

JNJ-A and JNJ-C were evaluated in single- and multiple-dose toxicity studies in rat and dog.

**Single dose.** Single-dose range-finding studies were conducted in male, Sprague-Dawley rats (Charles River, Kingston, NY) at doses of 1–2000 mg/kg, po (five per group). As part of the clinical chemistry assessment, fasting glucose measurements were made on the blood drawn 24 h after the administration of the single dose.

**Multiple dose.** Pancreatic histopathology was conducted at the end of a 5-day study in male and female Sprague-Dawley rats (Charles River) which were administered JNJ-A at 125 mg/kg/day, po (five per sex per group). The pancreas was fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin. Staining for insulin was conducted using immunohistochemical techniques with polyclonal antibody directed against swine insulin (Dako, Glostrup, DK).

Routine 4-week toxicity studies were conducted on JNJ-A at doses of 5, 25, and 75 mg/kg/day, po, in male and female Sprague-Dawley rats (Charles River) and 3 and 15 mg/kg/day, po, in male and female Beagle dogs (Marshall Farms, North Rose, NY). Numerous end points, including plasma glucose levels and histologic evaluation of pancreas, bone (distal femur and proximal
tibia), and Harderian gland, were assessed in separate groups of animals at the end of the 4-week dosing period as well as following a recovery period of 4 weeks. The histologic findings were quantified on a 5-point severity scale as normal or minimally, slightly, moderately, or severely affected. Plasma compound levels were determined at 24 h after administration of the last dose of JNJ-A using a liquid chromatographic-triple quadrupole mass spectrometric procedure. Data were analyzed using WinNonlin (Pharsight, Palo Alto, CA).

Mouse 5-Day Glucose Testing

To determine whether the reduction of insulin in the RINm5F cell assay predicted the hyperglycemic potential of a given compound, several compounds were selected for evaluation in a 5-day mouse study in which compound was administered and blood glucose was measured daily. The use of a 5-day model assured that exposure to drug was of sufficient duration to attain statistically significant elevations in blood glucose to differentiate diabetogenic from nondiabetogenic compounds. Male, CD-1 mice (Charles River), weighing between 18 and 24 g, were orally administered vehicle or test compound suspended in 0.5% hydroxypropylmethyl cellulose in water in a volume of 10 ml/kg. Prior to the administration of each daily dose, body weights were assessed, and blood glucose was measured with a One Touch Sure Step glucose meter (LifeScan, Inc., Milpitas, CA). When the blood glucose level reached the maximum value recorded by the meter (i.e., 600 ng/dl), the result was recorded as such. Thus, glucose measures in the groups administered the highest compound doses may actually exceed the stated values. On day 5, immediately prior to and at specified times after dosing, some mice at each dose level were sacrificed, and plasma and pancreas were collected for determination of drug levels.

RINm5F Cell Insulin Assays

RINm5F cells (ATCC CRL-11605 rat islet cell line) were grown in RPMI media (GIBCO 21878, Grand Island, NY), with supplements as specified by ATCC (Manassas, VA), at 37°C in 5% CO2. Three days prior to the addition of compound, cells were plated at 50,000 cells per well in a 96-well plate. On the day of the experiment, compounds were dissolved in dimethysulfoxide (DMSO) and diluted in media, such that the final concentration of DMSO in the assay was 0.1%.

Following a 24-h exposure to compounds, cells were washed and assessed for potential cytotoxic effect with a resazurin (Sigma, St Louis, MO) assay that quantifies the reduction of the substrate resazurin to resorufin by living cells over a 10-min incubation at 37°C. The resorufin formed was quantified with a TECAN SAFIRE II fluorometric reader (Raleigh, NC). The resazurin-containing buffer was removed, and the cells were washed with Hank’s balanced salt solution, containing calcium and magnesium, and lysed with lysis buffer (Pierce Biotechnology, Rockford, IL). After a 5-min shaking incubation, the plates were frozen at −80°C until used in the insulin ELISA. An aliquot of the cell extract was removed, and its insulin content was determined with a rat insulin ELISA kit (10-1124-10; Mercodia, Inc., Winston Salem, NC), according to the manufacturer’s directions. Insulin measurements were considered valid only when the resazurin cytotoxicity assay indicated a relatively high level of cell viability (i.e., >75%). Insulin levels were expressed as a percent of control to allow comparison among the results of experiments conducted on many different days.

Delta Opioid GTPγS Binding Assay

The agonist-stimulated GTPγS binding assay was performed essentially as previously described (Codd et al., 2006). Membranes from endogenous DOR-expressing NG-108 cells were purchased from Receptor Biology, Inc. (Baltimore, MD). A vial containing 1 ml of membrane was added to 15 ml cold media (GIBCO 21878, Grand Island, NY), with supplements as specified by the manufacturer, and aliquoted into 10 ml assay buffer. The receptor-containing membranes were preincubated in the assay buffer with wheat germ agglutinin–coated SPA beads (Amersham, Piscataway, NJ) at 25°C for 45 min.

### TABLE 1

<table>
<thead>
<tr>
<th>Plasma glucose (mg/dl)</th>
<th>JNJ-A</th>
<th>JNJ-C</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>98 ± 3.8</td>
<td>90 ± 9.1</td>
</tr>
<tr>
<td>10</td>
<td>103 ± 7.7</td>
<td>103 ± 7.7</td>
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<tr>
<td>100</td>
<td>119 ± 2.7</td>
<td>95 ± 3.7</td>
</tr>
<tr>
<td>500</td>
<td>146 ± 11.2</td>
<td>129 ± 6.7</td>
</tr>
<tr>
<td>1000</td>
<td>147 ± 12.4</td>
<td>122 ± 7.2</td>
</tr>
<tr>
<td>2000</td>
<td>—</td>
<td>236 ± 84.8</td>
</tr>
</tbody>
</table>

Note. The rats were fasted overnight prior to blood draw. The values shown are the means ± SEM. *N* = 5 per group

a Difference from control, *p* ≤ 0.01.
a Difference from control, *p* ≤ 0.05.

The SPA bead-coupled membranes were then incubated with 0.5mM [35S]-GTPγS (PerkinElmer, Boston, MA); each assay well contained 1 mg of SPA beads and 3–5 μg of membrane protein. The basal binding was defined as that taking place in the absence of added test compound, which was considered as 100%, with agonist-stimulated binding rising to levels substantially above this value. A range of concentrations of receptor agonists was used to stimulate [35S]-GTPγS binding. Both basal and nonspecific bindings were tested in the absence of agonist; the nonspecific binding determination included 10μM unlabeled GTPγS (Sigma). Radioactivity was quantitated on a Packard TopCount, and the percent stimulation was calculated as:

\[
\frac{(\text{test compound cpm} - \text{nonspecific cpm})}{(\text{basal cpm} - \text{nonspecific cpm})} \times 100
\]

EC50 values were calculated using GraphPad Prism (San Diego, CA).

#### Statistics

The statistical significance of differences in glucose levels in control- and drug-treated groups in the rat toxicology studies was determined with Dunnett’s (JNJ-A) or Dunn’s (JNJ-C) test, and the dose dependence of plasma glucose drug-treated groups in the rat toxicology studies was determined with Dunnett’s test compound cpm – nonspecific cpm

### RESULTS

#### Rat and Dog Toxicology Studies

The conduct of single-dose toxicology studies in rat revealed that JNJ-A as well as its sulfur analog JNJ-C (Fig. 2) elevated blood glucose levels at 24 h following oral administration (Table 1). This elevation exhibited a dose-dependent trend, reaching a level of 1.5 times that of the vehicle-treated group for JNJ-A at 1000 mg/kg and 2.6 times that of the vehicle-treated group for JNJ-C at a dose of 2000 mg/kg. Glucose levels were significantly different from those of the vehicle group at the 500 and 1000 mg/kg dose levels of JNJ-A and at the 2000 mg/kg dose of JNJ-C.

Histopathology conducted at the end of a rat 5-day dosing study on JNJ-A revealed changes in the pancreatic islets:
The islet cells contained large vacuoles, which generally contained eosinophilic material (Fig. 3A). Insulin immunostaining of control and affected pancreas revealed that, whereas most of the islet cells in controls were intensely positive for insulin, pancreatic insulin staining in rats administered 125 mg/kg/day JNJ-A was notably fainter. The pancreatic islets from these high-dose drug-treated animals were also larger.

Additional multiple-dose studies in rat and dog confirmed and extended these findings. Thus, when administered orally for 4 weeks, JNJ-A at a dose of 75 mg/kg/day elevated blood glucose to a level 1.9 times that observed in vehicle-treated rats (Table 2). Clinical signs indicative of compound-related diabetes mellitus included increased food consumption, coupled with decreased body weight and weight gain, as well as glucose in the urine and increased urine volume. Histopathology of the pancreas revealed minimal hypertrophy of pancreatic islet cells in 2 of 10 rats administered 25 mg/kg for 4 weeks and marked hypertrophy with vacuolation of pancreatic islet cells in all 11 rats administered 75 mg/kg/day for 4 weeks (Table 2). In addition, most also had minimal individual cell necrosis (apoptosis and oncotic necrosis). Transmission electron microscopy revealed that the vacuoles were membrane lined and appeared to be part of the endoplasmic reticulum and Golgi system. There was also reduced β-cell granule density.

A similar study conducted in dog did not reveal any significant elevation in blood glucose at the oral doses tested (3–15 mg/kg/day); however, at the highest dose, pancreatic islet cell hypertrophy (with vacuolation) was observed at a minimal level in one and at a moderate level in two drug-treated dogs. The drug exposures (expressed as the Cmax) at which pancreatic β-cell hypertrophy was observed in rat and dog were similar (i.e., 1155 ng/ml in rat and 1350 ng/ml in dog). Following a 4-week recovery period, rat plasma glucose returned to normal levels and pancreatic histopathology improved in both species (Table 2). In the rat, marked islet cell hypertrophy, noted at the end of the 4 weeks of dosing at the highest dose, recovered to moderate hypertrophy; and in the dog, hypertrophy at the minimal or moderate level completely

administered 125 mg/kg/day JNJ-A for 5 days (right panel). In contrast to the normal pancreas shown on the left, the pancreas from the drug-treated rat contained numerous islet cell vacuoles. The vacuoles contained insulin, although islets from drug-treated rats stained less densely for insulin than did islets from vehicle controls. The islets from the drug-treated rats were also larger. (B) Staining in the femur from a vehicle-treated rat (left panel) and a rat administered 75 mg/kg JNJ-A for 4 weeks (right panel). When compared with the femur from the vehicle-treated rat, the compound-affected femur exhibited discontinuity in the trabeculae, in the metaphysis, with more ossified trabeculae toward the mid shaft. Additionally, there was a widening of the zone of hypertrophied cartilage in the physis. (C) Staining in the Harderian gland from a vehicle-treated rat (left panel) and a rat administered 75 mg/kg JNJ-A for 4 weeks (right panel). When compared with the vehicle-treated rat, the treated rat exhibited glandular cell degeneration, including apoptosis/necrosis and regeneration with smaller basophilic cells often densely packed along the basement membrane.
<table>
<thead>
<tr>
<th>Dose (mg/kg, po)</th>
<th>Plasma^a glucose (relative to control)</th>
<th>Rat</th>
<th>Dog</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pancreas histopathology</td>
<td>Bone histopathology</td>
</tr>
<tr>
<td>3</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>5</td>
<td>No effect</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>15</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>25</td>
<td>No effect</td>
<td>Islet cell hypertrophy with vacuolation, minimal, 2/10</td>
<td>Normal</td>
</tr>
<tr>
<td>75</td>
<td>1.9×</td>
<td>Islet cell hypertrophy with vacuolation, marked, 11/11</td>
<td>Metaphyseal osteopenia (including trabecular discontinuity), 11/11 fracture, 5/11</td>
</tr>
<tr>
<td>75 rat or 25 dog; end of 4 week recovery period</td>
<td>Similar to control</td>
<td>Islet cell hypertrophy with vacuolation, moderate 4/4</td>
<td>Bone forming normally; residual metaphyseal trabecular discontinuity, 2/4; fracture, healing, 1/4</td>
</tr>
</tbody>
</table>

**Note.** — Indicates a dose that was not studied in that species or a measure that was not taken.

^a Blood samples taken 24 h after the last oral dose of JNJ-A.

^b NS, not significantly different from control.
normalized. In separate studies, similar effects were seen in both rat and dog following 5 days of dosing with JNJ-C (data not shown).

Changes in bone were also noted in rat studies, in which greater exposures were achieved relative to dog. In the 4-week study, metaphyseal osteopenia was noted in the femur and tibia of all of the high-dose (75 mg/kg) rats, with a discontinuity in the trabeculae between old trabeculae, which had normal amounts of bone, and newly formed trabeculae, which had notably decreased amounts of bone being laid down on the cartilage cores (Fig. 3B, Table 2). Numerous fractures were noted in the study. After the 4-week recovery period, although the effects of the bone change were still evident as a zone of trabecular discontinuity in the metaphysis, normal bony deposition was evident (Table 2). In separate studies, similar effects were seen after administration of high doses of JNJ-C.

In the rat study, degeneration and regeneration of the Harderian Gland was observed, the frequency and severity of which increased with the dose of JNJ-A (Table 2). The unilateral effect observed in the low-dose group occurred with a notably lower incidence, and severity and was generally associated with inflammation (such as might be expected from the prior bleeding procedure). The bilateral Harderian gland lesion in the affected compound-treated rats (Fig. 3C) was characterized by glandular cell degeneration, including apoptosis/necrosis, and by regeneration, with smaller more basophilic cells often densely packed along the basement membrane and having little secretory material.

**RINm5F Cell Insulin Assays**

The finding of pancreatic β-cell toxicity for these pharmacologically active compounds impelled a search for an in vitro assay that might be useful as a relatively high-throughput low-resource screen for the toxicity. Cyproheptadine, a marketed drug with structural similarities to the present novel tricycles (compare structures in Figs. 1 and 2, JNJ-A through E), and its metabolites have been extensively studied for their insulin-related properties in a model insulin-producing cell line, RINm5F (Miller and Fischer, 1990; Miller et al., 1993). Our initial studies confirmed concentration-dependent reduction of insulin in these cells by DMCPH epoxide (Fig. 4), yielding an ED$_{50}$ value of 17.9µM. In subsequent testing, the proprietary tricycles that produced diabetogenic effects and pancreatic β-cell hypertrophy in vivo (Tables 1 and 2) were tested in RINm5F cells. JNJ-A, its desethyl metabolite JNJ-B, and its sulfur analog JNJ-C concentration dependently reduced the insulin content of the cells (Fig. 4), with IC$_{50}$ values of 3.7, 0.67, and 2.1µM, respectively. The RINm5F cells were then used prospectively to assess the diabetogenic potential of other project compounds. As shown in Figure 5, at a concentration of 3.3µM, compounds of the tricyclic series (represented by circles) exhibited a range of effect on the insulin content of the cells, from having no effect (values near 100% of control) to having a profound insulin-reducing effect (lowest % control insulin values). Importantly, there was no relationship between the potency of compounds at DOR, as measured in the GTP$_{S}$ functional assay, and the effect of the compounds on the relative insulin content of RINm5F cells (Fig. 5).

Analysis of the structure-activity relationship among the tricycles that did not alter the insulin levels in RIN cells revealed two structural modifications associated with this property: (1) addition of a substituent at the piperidine nitrogen and (2) substitution of a tetrazole for the carboxylic acid diethylamide (examples of both types appear as open circles in Fig. 5). Another series of DOR agonists, the aryl-aryl-amines (AAA), represented by triangles in Figure 5 and including JNJ-F, was largely devoid of the insulin-reducing effect on RINm5F cells (notice the clustering of triangles to the right of the 75% value for percent control insulin in Fig. 5).

![FIG. 4. Reduction of insulin levels in RINm5F cells by desmethyl cyproheptadine (DMCPH) epoxide and several JNJ compounds. The points shown represent the mean ± SEM of triplicate determinations. The concentrations given in the legend are the IC$_{50}$ values calculated from the data shown.](https://academic.oup.com/toxsci/article-abstract/117/2/493/1640502)

![FIG. 5. Lack of relationship between DOR potency (EC$_{50}$, determined in the GTP$_{S}$ assay) and potency to reduce insulin (percent control insulin in RINm5F cells, determined at a 3.3µM compound concentration) for two structurally diverse sets of compounds, tricycles (open circles) and AAAs (triangles). Each symbol represents the data from an individual compound for each of the properties measured. The dashed lines represent a 50nM DOR EC$_{50}$ value (horizontal line) and an insulin level 75% of control (vertical line); the shaded portion of the graph highlights the area in which compounds have the desired profile, namely, highly potent DOR agonists that only weakly reduce RINm5F cell insulin.](https://academic.oup.com/toxsci/article-abstract/117/2/493/1640502)
Day 5 plasma and pancreatic levels of parent compound and metabolite (when samples of the metabolites were available to serve as a bioanalytical standard) were determined at times immediately prior to as well as at 1, 2, and 4 h following day 5 drug administration. Plasma levels of parent compounds were in the 2000–40,000 ng/ml range, whereas pancreatic drug levels were up to 30-fold higher (Table 4). Metabolite levels, where determined, were also higher (3- to 75-fold) in the pancreas than in the plasma.

**DISCUSSION**

*Diabetogenic Structure-Activity Relationship*

The unanticipated diabetogenic properties of the present DOR tricyclic compound series necessitated an evaluation of the possibility that the toxicity was DOR related. A review of the literature, however, brought attention to the structural similarity between the present tricyclic series and CPH, a serotonergic and histaminergic, nonopioidergic compound, which had already been shown to exhibit a similar pancreatic histopathology and blood glucose elevation. The pancreatic β-cell toxicity of CPH and its metabolites appears to be unrelated to the serotonergic or histaminergic properties of the compounds, as pharmacologically inactive metabolites also exhibit the toxicity. Furthermore, we determined that CPH and its metabolites were inactive at DOR (data not shown).

The structural similarity between CPH and the present tricyclic series included both the upper tricycle portion and a piperidine nitrogen located at the 4-position with respect to the attachment of the piperidine to the central ring of the tricycle. We extended this structure-activity relationship to other marketed drugs: The antihistamine desloratadine, a tricycle agonist structurally related to 4-DPMP, was shown to reversibly elevate blood glucose, induce pancreatic vacuoles, and deplete insulin in the rat (Otieno et al., 2008). Recently, AR-M100390, a DOR agonist structurally related to 4-DPMP, was shown to reversibly elevate blood glucose, induce pancreatic vacuoles, and deplete insulin in the rat (Otieno et al., 2008).

The importance of the DMCPH epoxide to the pancreatic profile of CPH (Chow et al., 1989) is of present interest given the structure of the diabetogenic tricycles. Thus, whereas an oxygen bridge is introduced into the CPH tricycle through a metabolic transformation, an oxygen or sulfur bridge is part of the core structure of the present DOR tricycles. Correspondingly, CPH is demethylated to yield the most potent moiety, DMCPH epoxide, whereas the present tricycles, as initially designed, were devoid of a substituent on the piperidine nitrogen. Thus, the early tricyclic structures could be viewed as already “optimized” with respect to the pancreatic outcome.

Importantly, structural modifications that averted the insulin depletion induced by the present series of DOR tricycles but...
FIG. 7. Blood glucose levels and body weight of mice administered hydroxypropylmethyl cellulose vehicle or the indicated JNJ compound daily for 5 days at the doses indicated. Blood glucose (left panels) and body weight (right panels) were measured immediately prior to dosing on each day. Shown are the means ± SEM (N = 5). *p < 0.05 compared with vehicle.
did not adversely affect DOR activity were identified. Overall, structure-activity analysis on the present tricyclic series revealed that the insulin-reducing effect of compounds in the series did not correlate with their DOR potency (see the scatter of the circles in Fig. 5). Moreover, medicinal chemistry efforts led to the identification and elaboration of a novel series of DOR agonists, the AAAs (triangles in Fig. 5), largely devoid of an insulin-reducing effect in RINm5F cells.

The importance of metabolism to CPH-induced toxicity is evidenced by species differences in CPH pancreatic toxicity, marked differences in toxicity following po versus ip. CPH administration and the reduction of toxicity by pretreatment with the metabolic inhibitor SKF-525A (Chow et al., 1988).

Furthermore, desmethyl CPH epoxide, the most potent CPH-related inhibitor of insulin synthesis (Fischer, 1997; Miller et al., 1993), is the predominant metabolite of CPH in rats.

### Table 3

<table>
<thead>
<tr>
<th>Compound</th>
<th>Delta opioid binding affinity $K_i$ (nM)</th>
<th>Delta opioid GTPγS $EC_{50}$ (nM)</th>
<th>RIN cell insulin level (% control at 3.3 μM)</th>
<th>Blood glucose elevation in mouse 5-day study</th>
<th>Fold increase in blood glucose (drug day 5/vehicle day 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JNJ-A</td>
<td>2.0</td>
<td>5.6</td>
<td>68</td>
<td>+</td>
<td>2.1$^a$</td>
</tr>
<tr>
<td>JNJ-B</td>
<td>74</td>
<td>14660</td>
<td>31</td>
<td>+</td>
<td>1.7$^a$</td>
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<tr>
<td>JNJ-D</td>
<td>1.7</td>
<td>35</td>
<td>13</td>
<td>+</td>
<td>1.9$^a$</td>
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<tr>
<td>JNJ-E</td>
<td>0.1</td>
<td>10.8</td>
<td>154</td>
<td>–</td>
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<tr>
<td>JNJ-F</td>
<td>22</td>
<td>111</td>
<td>101</td>
<td>–</td>
<td>0.90</td>
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</tbody>
</table>

$^a$Day 5 glucose level in drug-treated group (data taken from the highest dose tested, see Figs. 6 and 7) significantly different from that in vehicle-treated group at $p < 0.05$ level.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Plasma (ng/ml)</th>
<th>Pancreas (ng/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Parent compound</td>
<td>Metabolite</td>
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<tr>
<td>JNJ-A$^c$</td>
<td>0</td>
<td>0</td>
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<td></td>
<td>1</td>
<td>37,490</td>
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*Note.* Each compound was orally administered at 125 or 175 mg/kg, po for 5 days. Blood samples were taken for bioanalytical determination of compound levels prior to (0 time) or at the stated times after day 5 drug administration. $N = 2$ at each time point. NA, not available; ND, not determined.

$^a$Administered at 125 mg/kg, po, for 5 days.

$^b$Desethyl JNJ-A (JNJ-B).

$^c$Administered at 175 mg/kg, po, for 5 days.

$^d$Desethyl JNJ-B.

$^e$Desimidazolymethyl JNJ-E.
(Hucker et al., 1974; Iwaki et al., 1993) but is not formed in humans (Porter et al., 1975). Additionally, the low clinical dose of CPH administered to humans, 100 times lower than doses inducing pancreatic toxicity in rats, makes observation of the drug’s diabetogenic effect in humans unlikely, despite the observation of CPH inhibition of proinsulin synthesis in a study conducted at higher concentrations (0.5–100μM) in isolated human pancreatic islets (Jahr et al., 1981).

**Time Profile of the Diabetogenic Effect**

In the 5-day study in mice, conducted to follow up the RINm5F cell testing, it is difficult to assess to what extent differing pancreatic levels of test compound or metabolites contributed directly to the observed effects on blood glucose levels. The two compounds that did not elevate blood glucose in the mouse 5-day study were present at generally lower levels in the pancreas at the end of the study than were the diabetogenic compounds. Furthermore, compounds that elevated blood glucose in the study showed rising pancreatic levels of the administered compound (e.g., JNJ-B and JNJ-D) or of a hyperglycemic metabolite (e.g., JNJ-A’s metabolite JNJ-B) in the hours following oral administration of the parent compound. In contrast, pancreatic levels of the nonhyperglycemic compounds decreased across the time frame measured, and the nonhyperglycemic metabolite studied (the desimidazole-methyl-substituted compound JNJ-E) exhibited stable or slightly falling pancreatic levels. For only one compound, JNJ-F, the levels of compound in both the plasma and the pancreas prior to dosing on day 5 were below the limits of detection. It should be noted that the tissue levels of compounds are derived from extracts of the entire pancreas, although islets comprise only 1–2% of the mass of the pancreas (Bowen, 2002). Thus, the levels of compound actually present in the islets on day 5 are not known.

The pancreatic toxicity of CPH is a reversible effect. In insulin-producing cell lines, insulin synthesis was decreased by incubation of cells with micromolar concentrations of CPH but returned to normal within 48 h of CPH removal (Miller and Fischer, 1990). Discontinuation of CPH administration following its multiday dosing to rats similarly led to a restoration of normal pancreatic insulin levels (Rickert et al., 1975). The reversibility of the pancreatic effect of the present tricycles was also evident in the normalization of blood glucose levels and islet cell morphology in both rat and dog following recovery, during which time amelioration of bone and Harderian gland effects was also observed.

**Extension to PERK and WRS**

The constellation of effects observed here, notably the diabetogenic and epiphyseal dysplasia sequelae of compound administration, recapitulate the characteristic autopsy findings in WRS (Thornton et al., 1997; Wolcott and Rallison, 1972), an autosomal recessive disorder recently determined to be due to loss-of-function mutations in the eukaryotic translation initiation factor 2-α kinase 3 (EIF2αK3 or PERK) (Brickwood et al., 2003; Delepine et al., 2000). Phosphorylation of the initiation factor eIF2α, a key regulatory element in the initiation of protein synthesis, is controlled by four kinases, chief among which is PERK, with eIF2α phosphorylation resulting in reduced protein synthesis. High levels of PERK are found in exocrine organs, such as the pancreas, as well as in osteoblasts, which secrete type I collagen (Zhang et al., 2002), and PERK knockout mice display a similar array of deficits to those seen in WRS, including both permanent diabetes (Zhang et al., 2006) and impaired bone development (Wei et al., 2008).

Earlier studies had established that CPH administered to mice or rats induced a diabetogenic effect similar to that observed when PERK function is genetically impaired. Although RINm5F cells are well established as a model system in which to study the effect of CPH on pancreatic β-cells (Miller et al., 1993), only recently have RINm5F cell studies demonstrated that CPH increases phosphorylation of the initiation factor eIF2α (Hawkins and Fischer, 2004). Taken together with the previously demonstrated lack of effect of CPH on proinsulin mRNA (PPImRNA; Miller et al., 1993), the high levels of translationally uninitiated PPImRNA observed in the more recent study (Hawkins and Fischer, 2004) suggest that the diabetogenic effect of CPH may be at least partially due to altered regulation of protein synthesis by (PERK-mediated) eIF2α phosphorylation. Whether or not the tricyclic DOR agonists that produce the diabetogenic effects described here might similarly have a direct effect on PERK and/or eIF2α phosphorylation will also require further study.

The present study extends the well-studied pancreatic and diabetogenic effects of CPH-related compounds, identifying for the first time the concurrent induction of bone dysplasia by repeated administration of CPH-related compounds. Importantly, compound-induced bone dysplasia, like the pancreatic hypertrophy and diabetogenic effect, is reversible upon cessation of drug administration. These dual deficits on pancreas and bone are reminiscent of those exhibited by PERK disruption in WRS and PERK knockout mice, providing additional evidence for the involvement of PERK in the toxicity of CPH and related compounds. Although beyond the scope of the present work, further characterization of the bone deficits induced by CPH and related compounds is warranted.

In addition to diabetogenic/pancreatic and epiphyseal dysplasia findings, the present study also revealed compound-induced degeneration, with regeneration, of the Harderian gland in rat. Although the potential for a regulatory role of PERK/eIF2α in the Harderian gland has not yet been studied, it is tempting to speculate that, as another secretory gland, the Harderian gland also maintains control over its protein synthetic processes through PERK phosphorylation of the initiation factor eIF2α. Further study directed at this potential association seems warranted in light of the findings presented here.
Summary and Next Steps

The present series of studies describes the use of an in vitro, cell-based assay to detect the potential effect of test compounds on the insulin level of a pancreatic β-cell-derived line as well as the validation of these cell-based results in a multiple-day dosing study of compound effects on blood glucose in mice. Importantly, these studies demonstrate a lack of relationship between pancreatic toxicity and DOR activation. Furthermore, this tandem study paradigm enabled the identification of tricyclic DOR agonists that were devoid of the hyperglycemic effect characteristic of earlier compounds as well as of a novel nontricyclic DOR agonist series that did not reduce insulin levels or elevate blood glucose. Whereas previous studies of CPH-related compounds focused on diabetogenic effects (Fischer, 1997; Hawkins and Fischer, 2004; Otieno et al., 2008), the present study extends their findings to disruption of bone formation and to degeneration with regeneration of the Harderian gland. Although future studies will be required to corroborate the role of PERK in these newly identified sequelae following administration of CPH-like compounds, the present results open a path for the identification and progression of drug candidates, including DOR agonists, that lack diabetogenic potential.

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REFERENCES


