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Parallel strategies for the preparation and selection of liver-targeted glucocorticoid receptor antagonists

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Abstract—Libraries of mifepristone analogs, MP-Acids, were designed and synthesized to increase the chances of identifying GR antagonists that possess liver-selective pharmacological profiles. MP-Acids were uniformly potent GR antagonists in binding and in cell-based functional assays. A high throughput pharmacokinetic selection strategy that employs the cassette dosing of MP-Acids was developed to identify liver-targeting compounds. Thus, resource-intensive in vivo assays to measure liver-selective pharmacology were enriched with GR antagonists that achieve high concentrations in the liver.

While glucocorticoids mediate a multitude of functions in the body from anti-inflammatory responses to lipid, protein, and nucleic acid metabolism,[1] these steroid hormones are named for their primary role in modulating glucose metabolism. Functionally, glucocorticoids counter the actions of insulin by acting in the liver to inhibit glucose disposal and promote hepatic glucose production.[2] Patients with Cushing's disease produce an excess of the glucocorticoid cortisol and present a cluster of symptoms that mirror those seen in the metabolic syndrome.[3] In fact, overt diabetes develops in about 20% of these patients. The glucocorticoid receptor (GR) antagonist mifepristone **1** (Fig. 1) has been shown to improve glucose metabolism in these patients, reducing HbAlc levels (−40%),[3b] hepatic glucose output, and overall fasting blood glucose levels.[4] The extension of this treatment to patients with Type 2 diabetes mellitus (T2DM) has limited potential since the systemic blockade of GR produces compensatory responses that result in adverse outcomes.[5] For example, long-term generalized GR antagonism can cause either adrenal insufficiency, or overcompensation through the activation of the hypothalamic–pituitary–adrenal (HPA) axis resulting in adrenal hyperplasia and hypercortisolemia. Bile acid-mifepristone conjugate **3**, designed to provide liver-selective pharmacology, has been shown in animal models to effect dramatic reductions in glucose levels and HbA1c, while not activating the HPA axis.[6] As

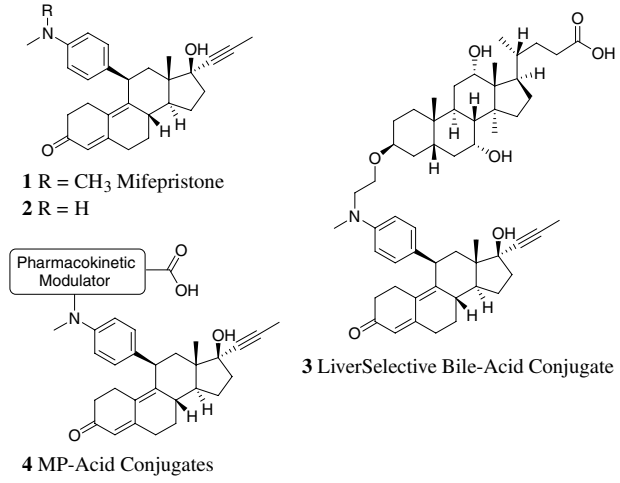

Figure 1. Steroid-based glucocorticoid receptor (GR) antagonists.

Keywords: Mifepristone; High-throughput pharmacokinetics; Parallel synthesis.
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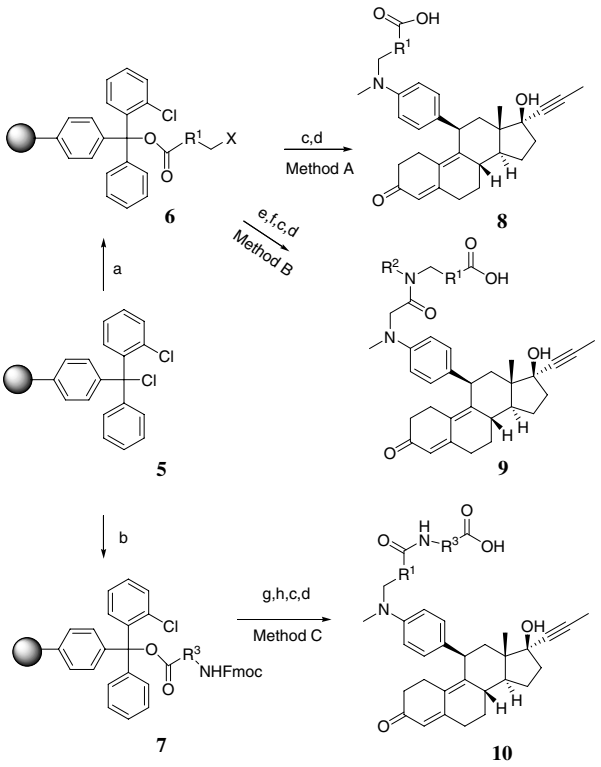
such, liver-targeted GR antagonists like **3** present a compelling and novel profile for the treatment of T2DM.

Researchers have invested significant effort devising schemes to target drugs to the liver. Bile acid conjugation[7] utilizes an 'address-and-message' strategy. Bile acids are tightly constrained to the enterohepatic loop passing through the intestine, to the portal vein, into the liver, through the bile duct and back to the intestine with extreme efficiency (enterohepatic recirculation).[8] These properties are due to the action of a series of transporter proteins. Conjugate **3** may engage these systems to some degree since substantial concentrations of **3** are sustained in the liver and high liver to blood ratios are observed. However, the vast majority of bile acid conjugates of **1** and bile acid conjugates of other GR antagonists that we tested did not possess similar liver-selective profiles. These findings may suggest that bile acid conjugation is not a general strategy for liver targeting and that the interpretation of the pharmacology of **3** within the 'address-and-message' framework may be overly simplistic.

As an alternative strategy, we proposed to (1) conjugate a range of pharmacokinetic modulators to mifepristone **1** using high throughput chemistry, (2) test these libraries of conjugates in in vitro GR binding assays and cell-based functional assays, and (3) select the best compounds for testing in animal models to evaluate liver-selective versus systemic GR antagonism. Central to the success of this strategy is the design of the pharmacokinetic modulators. A number of drugs show intrinsic liver-selective pharmacokinetics[9] and the serendipitous discovery of a suitable conjugate is not an unreasonable expectation. However, a feature common to many of these drugs is the presence of a carboxylic acid.[10] Indeed, the substrate specificity of many active transport systems implicated in the selective hepatic uptake of such drugs (organic ion transporting polypeptide-OATPs, organic anion transporting-OATs, and sodium taurocholate cotransporting polypeptide-NTCP) requires that substrates present an alkyl or aryl carboxylic acid. Accordingly, we devised high throughput chemistries to provide mifepristone conjugates **4** (MP-Acids). Additional benefits of conjugation to carboxylic acid-containing compounds included increased solubility, reduced *c*log *P*, and the stabilization of conjugates to metabolism at the C-11 aniline position (vide infra).

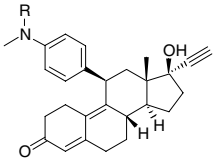
As with the design of **3**, attachment through the C-11 aniline of **1** was utilized to prepare conjugates since substitution at this position is well tolerated and enforces the antagonist conformation of the receptor.[11] A relatively large number (>150) and variety of MP-Acid conjugates **4** (Scheme 1) were rapidly prepared using a chlorotrityl linker, commercially available building blocks, three distinct solid phase chemistries, and high throughput reverse phase purification. Three general structural classes of conjugates emerged: hydrocarbon- (**11**, **14**, **15**, **17**), 2° amide- (**13**, **16**, **18**), and 3° amide-linked (**12**, **19**, **20**) acid conjugates (Table 1, Scheme 1).

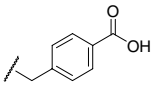
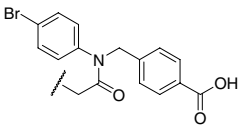
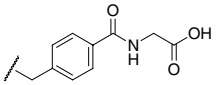
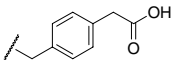
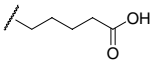
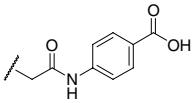
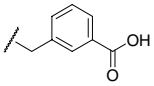
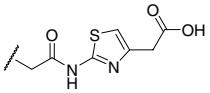
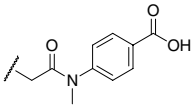
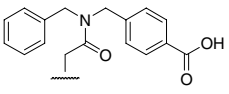


Scheme 1. General library synthesis methods to prepare MP-Acids **4**. Reagents and conditions: (a) XCH_2R^1COOH (2 equiv), *i*-Pr_2EtN (4 equiv), CH_2Cl_2, 2 h; (b) Fmoc-NHR^2COOH, (2 equiv), *i*-Pr_2EtN (4 equiv), CH_2Cl_2, 2 h; (c) **2** (1.2 equiv), *i*-Pr_2EtN (2 equiv), NMP, 60°C, 8 h; (d) CH_2Cl_2/AcOH/TFE (8:1:1), 4 h; (e) R^2NH_2, NMP, 60°C, 8 h; (f) bromoacetate (10 equiv), DICI (5 equiv), DMF, then *i*-Pr_2EtN (5 equiv), 4 h; (g) piperidine in DMF (20%), 20 min; (h) XCH_2R^1COOH (10 equiv), DICI (5 equiv), DMF, then *i*-Pr_2EtN (5 equiv), 8 h.

To determine GR (h-GR) binding the displacement of radiolabeled dexamethasone was measured. Functional activity was determined in a reporter cell line (h-GRAF) engineered to express h-GR and a glucocorticoid response element linked to a reporter gene encoding a secreted form of alkaline phosphatase. In addition, compounds were evaluated in their intended target cell, hepatocytes (HepTAT), stimulated with dexamethasone to increase the expression of GR-regulated proteins. The enzyme tyrosine aminotransferase was chosen as a convenient marker to determine the extent to which antagonists knock down the expression of key gluconeogenic enzymes.[12] All MP-Acids tested showed binding potencies comparable to that of **1** (representatives from each class: Table 1). This result is not unanticipated since the C-11 aniline substituents are relatively small and solvent exposed. In general, the functional activity (h-GRAF) of the MP-Acids is slightly lower than that of the parent **1** and may reflect a lower rate of passive diffusion into the cell. In agreement with this hypothesis is the observation that MP-Acids with larger C-11 aniline substituents trend toward reduced potencies. Activity measured in the HepTAT assay may be the most insightful measure of functional activity since several parameters are gauged. These cells balance active and passive uptake and secretion pathways since transport systems

Table 1. GR binding, GR cellular functional inhibition and inhibition of GR transcriptional activation



Compound (Synth Method)		h-GR[a]	h-GRAF[a]	HepTAT[a]
1		1.1	5	169
3		4.0	15	200
	**11 (A)**	1.8	12	52
	**12 (B)**	0.9	33	33
	**13 (C)**	0.6	90	36
	**14 (A)**	1.8	4.4	32
	**15 (A)**	4.8	15	127
	**16 (C)**	2.7	91.8	75
	**17 (A)**	3.0	30	112
	**18 (A)**	35	765	31
	**19 (A)**	0.5	21	64
	**20 (B)**	1.1	39	57

[a] All K_I values are determined in duplicate and are reported in nM.

are present. In addition, hepatic cells are metabolically competent and can reduce the amount of active drug in the cell. Strikingly, most MP-Acids were significantly more active than parent **1** or **3**, and little SAR could be discerned. As suggested by the potency observed in hepatocytes, MP-Acids demonstrate a resistance to metabolism. Compounds **11**, **13**, and **14** were shown to be, unlike parent **1**,[13] quite stable in rat liver microsome assays (Fig. 2).

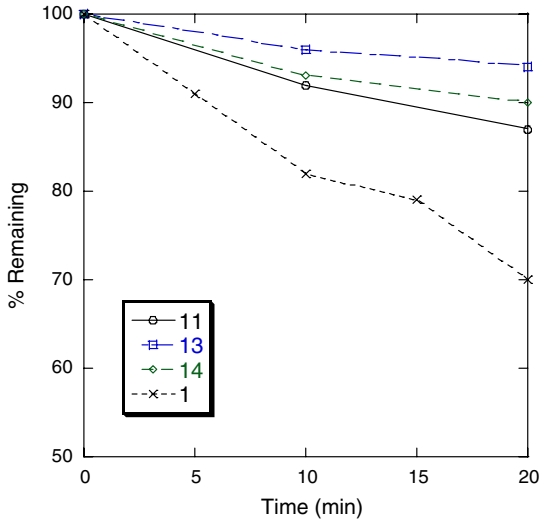
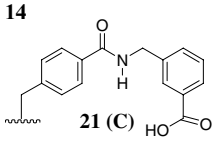
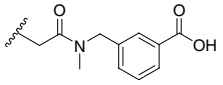
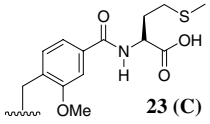
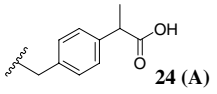


Figure 2. Metabolism of mifepristone **1** and MP-Acids **11**, **13**, and **14** at 10 μM in rat liver microsomes (0.25 mg microsomal protein/mL, 37 °C).

Since our compounds had all performed better than anticipated in the in vitro assays, we turned to additional measures to prioritize compounds for testing in our resource-intensive in vivo assays. Desiring potent GR antagonism in the liver, we devised a high-throughput pharmacokinetic (HTPK) selection protocol to estimate the liver levels of MP-Acids. A protocol was designed in which (1) MP-Acids were cassette dosed to Sprague–Dawley rats, (2) livers were harvested, and (3) the liver homogenates were analyzed by LC/UV to identify MP-Acids present in pharmacologically relevant concentrations. A key feature of our strategy was the inclusion of **3** in each cassette. The direct comparison of MP-Acid concentrations relative to **3** by AUC (area under the curve) was informative since all compounds possessed a strong chromophore at 304 nm resulting from the conjugated dione of the steroid core, and, thus, had similar extinction coefficients (Fig. 3a).

MP-Acids **11**, **13**, and **14** with **3** were used to develop our HTPK protocol. Extraction and sample handling techniques were optimized to provide comparable extraction efficiencies for all conjugates. LC/UV Trace 3b (Fig. 3) depicts the spiking of conjugates **3**, **11**, **13**, and **14** (1.5 mg/kg dose of each) into liver homogenate, followed by mixing, extraction, and LC injection. It is notable that the AUCs observed for each MP-Acid are comparable, and that little background from the extracted livers was observed at this wavelength (304 nm). In addition, the extraction efficiencies were >80% as judged by comparison to a standard curve generated for **3**. Several parameters were evaluated to develop a cassette dosing, sacking, extraction, and analysis protocol.[14] LC/UV Trace **3b** is representative of experiments in which a mixture of conjugates **3**, **11**, **13**, and **14** was orally dosed to Sprague–Dawley rats (1.5, 6 mg/kg total). Livers were harvested after 2 h, homogenated, and extracted. Results from several trials indicated that **11** and **14** were present in the liver in amounts comparable to **3** while **13** was not observed. Blood plasma was

Table 2. In vivo potency and liver selective pharmacology of GR antagonists

Compound (Synth Method)	% inhibition of pred response		
	TAT	Glycogen	Lymphocytes
1	101	77	104
3	72	86	9
11	106	94	123
13	48	47	40
14	95	101	117
 **21 (C)**	3	−12	−10
 **22 (B)**	20	9	−10
 **23 (C)**	−27	8	−4
 **24 (A)**	—	—	—

taken from the rats and evaluated in a similar manner. Only **11** and **14** were observed in plasma and estimates of liver/blood ratios were possible. However, the plasma levels for each were near the limits of detection, and moving forward, only liver levels of MP-Acids were interrogated. Greater than 150 MP-Acids were configured into cassettes and orally dosed to Sprague–Dawley rats (4–7 MP-Acids/cassette, **3** included in each cassette, 1.5 mg/kg/compd, 2 h time point).[15] Undosed cassette LC/UV traces were collected for comparison to those generated from the homogenated livers. Analysis indicated the presence of an additional four MP-Acids in the liver, in concentrations comparable to **3** (Table 2: **21**, **22**, **23**, and **24**).

We evaluated groupings of these MP-Acids in our rat prednisolone challenge (RPC) experiment to determine in vivo efficacy and liver-selective pharmacology (Table 2). In this experiment, Sprague–Dawley rats were given an oral dose of the systemically available GR agonist prednisolone (pred) (10 mg/kg) and a GR antagonist (100 mg/kg). GR-Mediated responses were monitored in the liver (increased levels of TAT and increased glycogen deposition) and the periphery (lymphopenia/anti-inflammatory effects). Accordingly, a liver-selective agent such as **3** modulates TAT expression and glycogen deposition without effecting lymphocyte levels, while a systemic agent like **1** affects all three markers. MP-Acids **11** and **14** displayed excellent in vivo efficacy knocking down TAT levels and decreasing glycogen deposition in the liver. However, neither was liver-selective since circulating lymphocyte levels remained high. MP-Acids **13** showed low systemic GR antagonism, but did not antagonize GR potently in the liver. These results were consistent with initial HTPK results in which **11** and **14** were found in liver and blood while **13** was absent in each. Compound **24** was not run due to its structural similarity to **11** and **14**, and in effect, its selection served as a positive internal control. MP-Acids **21**, **22**, and **23**, while not systemically active, were not active in the liver. Independent PK analysis showed that **23** was not present in the liver or blood. It is possible that this compound was an artifact peak seen in the LC/UV trace, since peaks/MP-Acids are identified by inference and not in an absolute manner. Interestingly, compounds **21** and **22** were observed in liver in high concentrations with high liver/plasma ratios (**24**: 37 μg/g in liver; liver/plasma ratio:17 at 1 h; **25**: 16 μg/g in liver; liver/plasma ratio:13 at 1 h) comparable to **3** (18 μg/g In liver;liver/plasma ratio:23 at 1 h). While **21** and **22** possess the desired pharmacokinetic profile, it is likely that high protein binding or other liabilities may dramatically reduce the activity of these compounds in the liver. In future rounds of HTPK selections and in vivo testing, additional filters may be added.

In conclusion, libraries of a new class of GR antagonists, MP-Acids, were designed to increase the chances of delivering compounds with liver-targeting properties. MP-Acids were shown to be potent GR antagonists in binding and cell-based functional screens. High-throughput pharmacokinetic (HTPK) selection strategies enriched in vivo liver-selective pharmacology assays with compounds that achieve high liver concentrations. Several MP-Acids were identified as potent, but systemic, GR antagonists in vivo. Further analysis

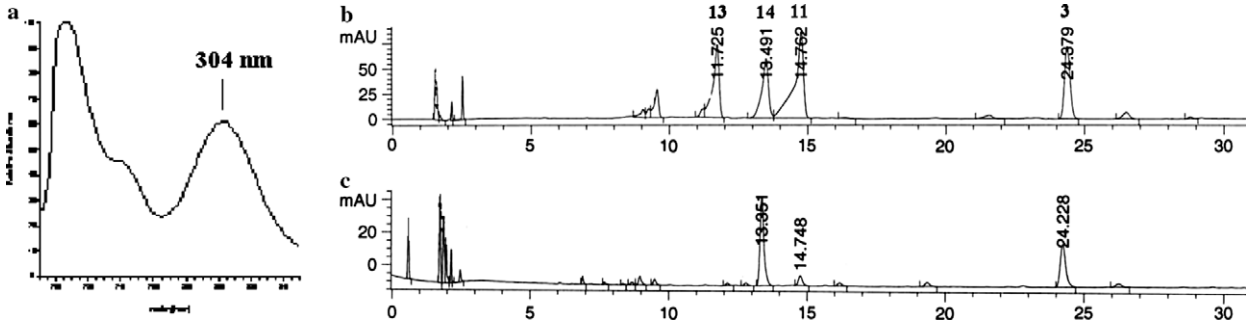


Figure 3. (a) MP-Acids possess a UV absorbance maximum of 304 nm (trace: 200–340 nm) resulting from the conjugated dione of the steroid core. (b) LC/UV trace (304 nm) of **3**, **11**, **13**, and **14** spiked into rat liver homogenate in equal amounts followed by extraction. (c) LC/UV trace of extracted liver homogenate for oral cassette dosing of compounds **3**, **11**, **13**, and **14** (1.5 mg/kg each, 2 h).

of these and other MP-Acid libraries will be reported in due course.

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12. For in vitro assays, all compounds displayed full antagonist activity in blocking the agonist response of dexamethasone.
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14. Oral dosing of **3** (10 mg/kg) to Sprague–Dawley rats resulted in liver levels of 8 μg/g at 1 h (independent PK determination). The detection limit for **3** using LC/UV analysis at 304 nm was well below this regime (<10 ng). With extraction recovery efficiencies well over 80%, we targeted the extraction of 0.5 g of liver and dosed **3** and MP-Acids at 1.5 mg/kg (total dosing around 10 mg/kg).
15. Bile acid conjugate **3** was added to each MP-Acid cassette (**4**–**7** compounds) such that all compounds were at 1.5 mg/kg. The cassettes were formulated (5% EtOH, 10% Peg-400 vehicle with a dilute NaOH solution (0.1 N) added dropwise to facilitate solubility) and orally dosed to Sprague–Dawley rats. Rats were sacrificed after 2 h and livers were taken (livers were prepared as a 9:1 PBS/liver homogenate). Homogenate (5 mL) was added to a 50 mL centrifuge tube and MTBE (10 mL) was added. The mixture was vortexed (45 s) and samples were partitioned using centrifugation (5 °C, 2500 rpm, 10 min). Organics were separated and concentrated with a stream of nitrogen (no heat). A 1:1 DMSO/H_2O solution was added (150 μL) and samples were filtered (0.45 μm hydrophilic filters). Samples were analyzed by LC/UV (100 μL injection, Phenomenex Luna 5μ C18 (2) 100 A, 250 × 4.6 mm column, 304 nm, 5–35% over 5 min, 35–52% over 15 min, then 5 min to 100%, CH_3CN, H_2O with 0.1%TFA). LC/UV traces of liver homogenate from all cassettes showed the presence of **3** and LC/UV traces of undosed cassettes were collected for comparison.