OXIDATIVE METABOLISM OF A REXINOID AND RAPID PHASE II METABOLITE IDENTIFICATION BY MASS SPECTROMETRY

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ABSTRACT:

LGD1069 (Targretin), a retinoid "X" receptor-selective ligand, or rexinoid, is in clinical trials for treating cancer. Biologically-active oxidized LGD1069 metabolites have been observed in patient plasma samples, making corresponding structural characterizations necessary. Formation of multiple metabolite isomers in vivo has created technical challenges in metabolite structural analysis; however, mass spectrometry (MS) was able to pinpoint two sites of Phase I metabolism. A carbon-13 deuterated analog was used as an isotopic marker to probe Phase II metabolism of LGD1069. Rats were orally gavaged with an equimolar mixture of LGD1069 and \([^{13}\text{C}_2\text{H}_3]\text{LGD1069}\), then anesthetized prior to bile-duct cannulation. Bile was collected for 7 hr, extracted, and concentrated. Recovered metabolites were analyzed by narrow-bore, gradient liquid chromatography (LC) with negative ion, electrospray ionization MS detection. When resultant total ion chromatograms were interrogated for mass spectra exhibiting isotope clusters separated by 4 daltons, 13 such clusters corresponding to Phase II LGD1069 metabolites of nine different molecular weights were detected. Acyl glucuronide and taurine conjugates of both parent compound and hydroxy-LGD1069 were observed. The sulfate and taurine conjugates of o xo-LGD1069 were also identified, as were 6,7-di hydroxy-LGD1069 taurine, LGD1069 ether glucuronide, and a secondary conjugate (taurine) of the latter. Identities of selected conjugates were confirmed by MS/MS. The results of this study demonstrate that when combined with traditional GC/MS and MS/MS data, the isotopic cluster technique can provide powerful selectivity in identifying numerous Phase II drug metabolites during a single LC/MS analysis.

LGD1069, 4-[1-(3,5,5,8-pentamethyl-5,6,7,8-tetrahydro-2-naphthalenyl)-1-ethenyl]-benzoic acid, is termed a rexinoid, indicating it is a ligand which binds selectively to the retinoid "X" subclass of retinoid receptors, as opposed to the retinoic acid subclass. The biology associated with this class of compounds has been reviewed extensively elsewhere (1). LGD1069 (Targretin) is in clinical trials for treatment of various oncologic conditions including lung cancer. It is a very well tolerated drug in both humans and rats (2). At least two metabolites observed in human plasma retain significant biological activity toward retinoid receptors in vitro (4). In the rat, production of these metabolites is induced following 7 days of oral treatment at 100 mg/kg/day (5). After HPLC\(^1\) isolation of these metabolites from rat microsome incubations, molecular weight information from ESI mass spectrometric analysis indicated mass increases of 14 and 16 daltons, suggesting the metabolites contained oxo and hydroxyl moieties, respectively. These metabolites exhibit identical HPLC retention times as the circulating active metabolites in patients and are the major ones produced by human microsomes (6). Systemic exposure of patients to the hydroxylated metabolite seems to be approximately twice that of parent compound while exposure to o xo-LGD1069 is similar to that of parent.\(^2\)

Although not a retinoic acid derivative, LGD1069 contains one carboxylic moiety and is structurally similar to other retinoid-receptor ligands such as the RAR-selective Am-80 and TTNPB (7, 8). Unlike these ligands, however, LGD1069 has a C-3 methyl substitution on its tetrahydro-naphthalenyl moiety which is believed to contribute to its RXR affinity (Structure A). Am-80 is metabolized by rats specifically at the C-6 and C-7 positions of the tetrahydro-naphthalenyl moiety, presumably using cytochrome P450 (7). Based on its structural similarity to Am-80, LGD1069 might be oxidized at the same positions. This hypothesis was explored in the first part of the present study using rat microsome-derived HPLC fractions, with the primary objective of characterizing the oxidized position accounting for the active circulating metabolites of LGD1069 in humans. The structure of LGD1069 contains a high degree of symmetry and chromatographic efforts to isolate pure metabolite isomers were unsuccessful. Nevertheless, gas chromatography/mass spectrometry was successful in structurally characterizing several Phase I metabolites. LC/MS has emerged as an effective method for identification of drug metabolites in crude extracts of biological matrices such as bile. To facilitate identification of Phase II metabolites of LGD1069, an equimolar mixture of a carbon-13, deuterated analog, and unlabeled LGD1069 was administered to rats. The mass spectra of LGD1069 metabolites would then be characterized by unique stable isotope

\(^1\) Abbreviations used are: HPLC, high-performance liquid chromatography; LC, liquid chromatography; RAR, retinoic acid receptor; RXR, retinoid "X" receptor; MS, mass spectrometry; PFB-Br, pentfluorobenzyl bromide; DIEA, diisopropylethylamine; BSTFA, bis(trimethylsilyl)trifluoroacetamide; TMS, trimethylsilyl; MOX-HCl, methoxamine hydrochloride; ESI, electrospray ionization; EI, electron ionization; NCI, negative ion electron capture ionization; CID, collision-induced dissociation; TIC, total ion chromatogram; SER, smooth endoplasmic reticulum; Da, daltons.

\(^2\) Data on file, Ligand Pharmaceuticals.

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mm DB-1, 0.25 equimolar mixture of LGD1069 and [13C2H3]LGD1069 suspended in super-

300 g) were orally gavaged with a total of 100 mg/kg of an

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Materials and Methods

Chemicals. LGD1069 (MW 348) and its stable isotope labeled analog, [13C2H3]LGD1069 (MW 352), were synthesized and purified (to ≥ 99% purity) as described previously (8,10). HPLC solvents were purchased from Fisher Scientific (Fairlawn, NJ). Dexamethasone and NADPH were purchased from Sigma Chemical Co. (St. Louis, MO). PFB-Br, DIEA, BSTFA, and MOX-HCl were purchased from Aldrich Chemical Co. (Milwaukee, WI).

Instrumentation. An analytical column (Microsorb-MV 5 μm, C-18, 4.6 × 250 mm) from Rainin Instrument Co., Inc. (Woburn, MA) was used for collection of fractions containing hydroxy- and oxo-LGD1069. A 5 m × 0.25 mm DB-1, 0.25 μm film thickness, capillary GC column (J&W Scientific, Folsom, CA) was interfaced with a Finnigan SSQ-70 mass spectrometer, used for acquisition of EI and NCI mass spectra. The injector temperature was maintained at 275°C and the transfer line at 300°C. Samples (1 μl) were injected at an initial column temperature of 150°C followed by a linear ramp of 15°C/min to 300°C. For NCI, methane was used as the reagent gas. Spectra were acquired over m/z 50–700 at 1 sec/scan. For LC/MS, a 2.0 × 150 mm Ultramex 3 μm, C-18 column was interfaced with a Sciex API III mass spectrometer. Spectra were acquired over m/z 100–800 at 3 sec/scan with a spray voltage of −3200 V and an orifice voltage of −60 V. CID spectra were acquired over m/z 50–550 with a pressure of argon in the second quadrupole equivalent to 200 × 1013 molecules/cm2 and a collisional offset potential of −30 V.

Biological Experiments. For Phase I metabolite production by rat microsomes, hepatic microsomes were isolated from male Sprague-Dawley rats (~300 g) which had received five daily doses of dexamethasone (50 mg/kg/day, ip). Microsome incubations were performed with a starting concentration of 100 μM LGD1069 or 100 μM [13C2H3]LGD1069 for 4 hr in the presence of a NADPH-generating system. For Phase II metabolism production, two fed male rats (~300 g) were orally gavaged with a total of 100 mg/kg of an equimolar mixture of LGD1069 and [13C2H3]LGD1069 suspended in superfine sesame oil (Crorda Inc., Parsippany, NJ). The rats were then anesthe-
tized (80 mg/kg ketamine and 4 mg/kg xylazine, ip) and their bile ducts were cannulated (0.025 mm OD × 0.012 mm ID tubing, Braintree Scientific, Inc., Braintree, MA). Ketamine and xylazine were administered as needed to maintain anesthesia for up to 7 hr of bile collection. Two-hundred μl of bile from the first and last (5th or 7th) hr of collection were extracted with 5 volumes of methanol. After chilling to −78°C and centrifuging at 4°C, the supernatants were dried by evaporation in vacuo and reconstituted in 10 mM ammonium acetate (NH4OAc)/glacial acetic acid (HOAc), 1000:1 (v/v). Narrow-

row-bore, gradient LCMS used a solvent gradient of 20% CH3CN/20 mM NH4OAc, pH 6.0 to 80% CH3CN in 20 min and then to 100% CH3CN in 10 min. The solvent flow rate was 200 μl/min. Mass detection was in the negative ion mode. Multiview 68K (v 1.1.1) isotope cluster analysis software (PE Sciex, Ontario, Canada) was used to interrogate a total ion chromatogram for mass spectra exhibiting characteristic ion doublets separated by 4 amu. One advantage of the isotope cluster technique was that LGD1069 metabolites could be uniquely identified for structural analysis by the appearance of ion doublets in their mass spectra making analysis of control bile unnecessary. For GC/MS, PFB-Br was used to derivatize carboxylate moieties, BSTFA was used to derivatize hydroxyl moieties, and MOX-HCl was used to derivatize oxo moieties.

Results

Two major circulating metabolites of LGD1069 were hypothesized to be oxo- and hydroxy-LGD1069 based on previous loop-injection ESI-MS analyses which revealed molecular weights of 362 Da and 364 Da, respectively. Since these analyses did not provide structural information, GC/MS and MS/MS were used in the present study to determine the sites of oxygen substitution. For analysis of the hydroxylated metabolite by EI and NCI GC/MS, derivatization to its TMS ether was chosen to aid in chromatography and elicit structurally-informative fragmentation characteristics. For both metabolites, the carboxylate moiety was derivatized with a polyfluorinated reagent to facilitate gas chromatography, promote fragmentation, and provide the derivatives with electron-capturing properties for NCI conditions. Further derivatization of the oxo metabolite with MOX-HCl was used to increase the mass by a diagnostic 29 amu to confirm presence of the putative oxo moiety by conversion to its corresponding methylxime.

The EI mass spectrum of the PFB-ester, TMS-ether derivative of the hydroxy metabolite exhibited a molecular radical cation at m/z 616 and a base peak of m/z 511 (fig. 1). The base peak ion suggested a stabilized cation resulting from neutral loss of TMSOH from the [M-CH3]+ fragment ion (m/z 601).

The base peak of m/z 511 suggested a stabilized cation resulting from neutral loss of TMSOH from the [M-CH3]+ fragment ion (m/z 601).
(parent cleavage ion), m/z 317 ([M-PFB-CO2]−) and m/z 181 (PFB, C3H5F3). The NCI spectrum contained an [M-181]− ion at m/z 361 (M-PFB). Analyses of derivatized [13C2]H3-labeled metabolites produced mass spectra consistent with these assignments. NCI analysis of the MOX derivative of oxo-LGD1069 revealed the expected mass increase of 29 amu ([M-PFB]−, m/z 390), consistent with the oxo assignment. Although absolute enantiomeric and positional configurations could not be defined for specific components in the HPLC fractions, all Phase I metabolite data were consistent with oxidative metabolism of LGD1069 resulting in substitution at the C-6 or C-7 position.

ESI MS/MS was used to analyze underivatized hydroxy-LGD1069 to provide additional data on the positions of hydroxyl substitution. The most abundant product ion under CID conditions appeared at m/z 83 (fig. 2). Formation of this ion is likely initiated with a charge site on the hydroxyl moiety at C-6 or C-7. Charge-directed fragmentation results in ring opening and rearrangement to form 3-methyl-2-butenal.

CID-mediated formation of m/z 83 ion is likely initiated with a charge site on hydroxy moiety at C-6 or C-7. Charge-directed fragmentation results in ring opening and rearrangement to form 3-methyl-2-butenal.

In the TIC from LC/MS analysis of a rat bile sample (7 hr post-dose), many peaks were observed (fig. 3, top). Using an isotope cluster analysis program, the TIC was interrogated for mass spectra exhibiting ion doublets separated by 4 amu (fig. 3, bottom). This cluster analysis program enabled rapid detection of 13 isotope clusters that were all consistent with expected molecular ions for 13 different Phase II LGD1069 metabolites. Representative mass spectra, corresponding to the two peaks labeled B and H in fig. 3B, illustrate the utility of the isotope cluster technique in detecting drug metabolites in a crude extract of a complex biological matrix such as bile (fig. 4). The identities of the various metabolites were assigned based on their mass difference from parent compound (table 1). Metabolite peaks labeled in small letters in fig. 3B and table 1 (a, e, h, h’) are isomers of the corresponding capital-lettered metabolite peaks, h and h’ presumably being acyl-migrated glucuronides of the parent. All bile samples, early and late from two animals, were qualitatively similar when analyzed by LC/MS.

After molecular weight elucidation of many LGD1069 metabolites from a single LC/MS analysis, ESI MS/MS was used to confirm the identities of several taurine and glucuronide conjugates. Negative ion CID spectra of the m/z 454 and m/z 458 ions revealed product ions expected from the taurine conjugate of LGD1069 (m/z 124, H3N-CH2-CH2-SO3−; m/z 107, CH2=CH-SO3−; m/z 80, SO3−, fig. 5). CID spectra of the taurine conjugate of hydroxy-LGD revealed the same product ions from the precursor ions, m/z 470 and m/z 474 (data not shown). The CID spectrum of m/z 523 contained abundant [M-H-176]− (LGD1069, m/z 347) and [M-H-176-44]− (LGD1069-CO2, m/z 303) ions, providing support for its identity as the acyl-glucuronide of LGD1069 (fig. 6). Two metabolites with substantially different retention times (3.36 and 6.83 min) each exhibited ion...
clusters of m/z 539 and m/z 543, consistent with glucuronidated hydroxy-LGD1069. The CID spectra of both metabolites contained [M-H-176]⁻ ions at m/z 363, consistent with corresponding aglycones (fig. 7). Under reversed phase HPLC conditions, the 6- or 7-ether glucuronide of LGD1069 would be expected to elute earlier than the acyl glucuronide owing to two carboxylate moieties located on opposite ends of the molecule. Thus, the metabolite eluting at 3.36 min was assigned an ether linkage. In addition to the ion at m/z 363, the CID spectrum of this metabolite contained the [M-H]⁻ parent ion at m/z 539 and an [M-H-44 –18]⁻ ion at m/z 477, consistent with loss of CO₂ and H₂O (fig. 7, top). The CID spectrum of the acyl-glucuronide of hydroxy-LGD1069, eluting later at 6.83 min, lacked these ions and showed very little fragmentation other than the aglycone, [M-H-176]⁻, at m/z 363 (fig. 7, bottom). From these observations, it appears that loss of 176 is quite facile for the acyl linkage but is not as facile for the ether-linked conjugate. Thus, loss of 44+18 was observed in addition to the loss of 176 in the CID spectrum of the ether-linked glucuronide of hydroxy-LGD1069.

**Discussion**

The hydroxy and oxo metabolites of LGD1069 represent major circulating metabolites in humans. These metabolites were structurally characterized by positive and negative ion GC/MS analysis as derivatives as well as negative ion MS/MS of underivatized hydroxy-LGD1069. Mass spectrometric analysis revealed oxidation at C-6 or C-7, and based on HPLC elutions as double peaks, both isomers were concluded to exist. However, absolute differentiation between substitution at the 6- versus 7-positions was not achieved, nor were assignments of absolute stereochemistries. Since the hydroxyl substituents can exist in either equatorial or axial conformations relative to the cyclohexene ring, two enantiomers were possible for the C-6 and C-7 positional isomers of hydroxy-LGD1069. Only one isomer was possible for 6- and 7-oxo-LGD1069 since the oxo moieties exist in a planar conformation.

In addition to accounting for ions observed, the absence of other ions in the EI mass spectra provided support for the assignment of
oxygen substitution at C-6 and C-7. For example, if hydroxyl substitution had been at one of the primary, gem-dimethyl carbons, the EI spectrum would be expected to exhibit an abundant ion at m/z 103 (TMS-OCH$_2$), based on fragmentation behavior of the methyl-ester, TMS-ether derivative of 20-hydroxyeicosanoic acid (11). On the other hand, if hydroxylation had occurred at the stable-label-containing methyl group at C-3, the corresponding hydroxylated metabolite would be expected to exhibit an abundant ion at m/z 106 (TMS-O$^{13}$C$_2$H$_2$). No such ions were observed, consistent with hydroxylation occurring at C-6 and C-7 versus other positions in the molecule. Once the sites of oxidation on the Phase I metabolites were characterized, the Phase II conjugation reactions were presumed to occur at these, and not other, sites. The carboxylate moiety on both the oxo and hydroxyl metabolites served as an additional site for conjugation reactions. Based on molecular weights identified and not including acyl-migrated glucuronides, 28 different Phase II metabolite structures are possible if all C-6- and C-7-substituted Phase I metabolite isomers are produced (fig. 8).

The unusual di-conjugate metabolite, containing taurine and an ether-linked glucuronic acid moiety, is of interest owing to its origination from one or more unknown sequences of distinct metabolic steps, taurine conjugation in the mitochondria, and both glucuronidation and a cytochrome P450-mediated step at the SER. A priori, since the glucuronic acid in the metabolite is ether-linked, hydroxylation must precede glucuronidation. Either the hydroxy metabolite or the glucuronide conjugate would need to be taken up into mitochondria and activated to a coenzyme-A thioester (12) if taurine conjugation occurs last. Alternatively, if taurine conjugation occurs first, the taurine conjugate itself would need to serve as a substrate for two later SER-mediated metabolic steps. This sequence, which is depicted in fig. 8, is possible since polarity of the taurine moiety would not affect the lipophilic portion of the molecule needing access to cytochrome P450 for oxidation to occur at C-6 or C-7. Secondary conjugation with glucuronic acid might then occur readily, particularly if the hydroxylation and glucuronidation steps are coupled, as was recently reported for the substrate, salicylamide (13).

No unconjugated di-hydroxy, di-oxo, or oxo, hydroxy metabolites of LGD1069 were observed. However, through a comprehensive search for conjugated metabolites using LC/MS and the isotope clus-
The technique, indirect evidence for production of both a di-hydroxy metabolite (taurine conjugate “C”) and an oxo, hydroxy metabolite (sulfate conjugate “G”) was obtained (table 1, fig. 8). By combining the use of GC/MS, LC/MS, and MS/MS, many metabolites of LGD1069 in the rat have been characterized. With a high degree of selectivity, the molecular weights of at least 13 Phase II metabolites were rapidly identified from one chromatographic analysis using LC/MS and the isotope cluster technique. Although the biologically-active oxo and hydroxy metabolites are produced and circulate systemically, production of the additional metabolites described herein remains to be investigated in man.

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References


