Bioactivation of α,β -Unsaturated Carboxylic Acids Through Acyl Glucuronidation[§]

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ABSTRACT

After oral administration to monkeys of [¹⁴C]GDC-0810, an α,β -unsaturated carboxylic acid, unchanged parent and its acyl glucuronide metabolite, M6, were the major circulating drug-related components. In addition, greater than 50% of circulating radioactivity in plasma was found to be nonextractable 12 hours post-dose, suggesting possible covalent binding to plasma proteins. In the same study, one of the minor metabolites was a cysteine conjugate of M6 (M11) that was detected in plasma and excreta (urine and bile). The potential mechanism for the covalent binding to proteins was further investigated using in vitro methods. In incubations with glutathione (GSH) or cysteine (5 mM), GSH and cysteine conjugates of M6 were identified, respectively. The cysteine reaction was efficient with a half-life of 58.6 minutes ($k_{\text{react}} = 0.04$ 1/M per second). Loss of 176 Da (glucuronic acid) followed by 129 Da (glutamate) in mass fragmentation analysis of the GSH adduct of M6 (M13) suggested the glucuronic acid moiety was not modified. The conjugation of N-glucuronide M4 with cysteine in buffer was >1000-fold

slower than with M6. Incubations of GDC-0810, M4, or M6 with monkey or human liver microsomes in the presence of NADPH and GSH did not produce any oxidative GSH adducts, and the respective substrates were qualitatively recovered. In silico analysis quantified the inherent reactivity differences between the glucuronide and its acid precursor. Collectively, these results show that acyl glucuronidation of α,β -unsaturated carboxylic acids can activate the compound toward reactivity with GSH, cysteine, or other biologically occurring thiols and should be considered during the course of drug discovery.

SIGNIFICANCE STATEMENT

Acyl glucuronidation of the α,β -unsaturated carboxylic acid in GDC-0810 activates the conjugated alkene toward nucleophilic addition by glutathione or other reactive thiols. This is the first example that a bioactivation mechanism could lead to protein covalent binding to α,β -unsaturated carboxylic acid compounds.

Introduction

Approximately 80% of all breast cancers express and are dependent on the estrogen receptor (ER) for tumor growth and progression (Jemal et al., 2011). Despite the effectiveness of available hormonal therapies such as tamoxifen, aromatase inhibitors (e.g., anastrozole, letrozole, and exemestane), and full ER antagonists/degraders (e.g., fulvestrant), many patients develop resistance to these agents and, hence, require further treatment (Di Leo et al., 2010; Miller et al., 2010; Van Tine et al., 2011; Baselga et al., 2012). GDC-0810 (Fig. 1), an α,β -unsaturated carboxylic acid, is a novel selective estrogen receptor alpha (ER α) antagonist and inducer of ER α degradation, which has potential to be used as an oral anticancer drug for estrogen receptor-positive breast cancer as either a single agent or in combination with other anticancer drugs.

While investigating GDC-0810 as a candidate molecule, several in vivo pharmacokinetics and metabolism studies were performed. Radioactivity extraction recovery was low (<50%) in plasma of monkeys after oral administration of [¹⁴C]GDC-0810. Extensive organic

extraction of plasma proteins suggested that the radioactivity was covalently bound to proteins. In monkey plasma, the identification of M11, the cysteine conjugate of M6, provided us with a clue that a nucleophile could form an adduct with M6 through Michael addition to the β -carbon of the major circulating metabolite. To further elucidate the mechanism for the protein covalent binding, in vitro experiments were conducted to answer the following questions: 1) How efficient is the Michael addition of a nucleophile to the major circulating metabolite M6? 2) Does Michael addition occur for GDC-0810 or other metabolites? 3) Was there any direct replacement of the acyl glucuronide by a nucleophile? 4) Was a Schiff base formed with an amino group from an acyl-migration product, leading to formation of an adduct? 5) Was there any bioactivation by cytochrome P450 (P450) enzymes to form reactive metabolites that could be trapped by glutathione (GSH)? 6) Was any AMP or coenzyme A (CoA) adduct formed in hepatocyte incubations? Herein, we discuss these experiments and findings that can be related to protein covalent binding.

Materials and Methods

Chemicals and Reagents. GDC-0810, its acyl glucuronide (M6), and its N-glucuronide (M4) were synthesized and characterized at Genentech

All authors are Genentech employees when the research was conducted.

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ABBREVIATIONS: CoA, coenzyme A; ER, estrogen receptor; ER α , selective estrogen receptor alpha; GSH, glutathione; HPLC, high-performance liquid chromatography; LC-MS, liquid chromatography/mass spectrometry; LSC, liquid scintillation counting; LUMO, lowest unoccupied molecular orbital; MS, mass spectrometry; MS/MS, tandem mass spectrometry; m/z , mass-to-charge ratio; P450, cytochrome P450.

Fig. 1. Metabolic pathways and proposed bioactivation mechanism of GDC-0810.

(South San Francisco, CA). [¹⁴C]GDC-0810 was synthesized by Selcia (Essex, UK) with the specific radioactivity of 8.8 kBq/mg.

Liver microsomal preparations and cryopreserved hepatocytes were purchased from In Vitro Technologies (Melbourne, AU). Acetonitrile and methanol were purchased from EMD Chemicals (Gibbstown, NJ). Ultrapure high-performance liquid chromatography (HPLC) water and formic acid were purchased from J.T. Baker (Center Valley, PA). Ammonium formate and ammonium hydroxide solution were purchased from Fluka (St. Louis, MO). Sodium citrate, GSH, cysteine, and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Monkey Plasma Collection, Extraction, and Metabolite Profiling. Plasma samples were collected from cynomolgus monkeys after 50 mg/kg oral administration of [¹⁴C]GDC-0810. Approximately 1.5 g of each plasma sample was treated with 3× volume of 0.1% formic acid in acetonitrile for protein precipitation. Samples were then mixed by vortex for 5 minutes and sonicated for 10 minutes at room temperature. Supernatants were transferred to a new set of tubes after centrifugation at 2700g for 30 minutes. Extraction recoveries were determined by liquid scintillation counting (LSC). The sample-extraction recovery of radioactivity was assessed by comparing the total radioactivity in the processed samples with the total radioactivity in unprecipitated plasma. After extraction, the weight of the supernatant was measured, and an aliquot of the supernatant was counted for radioactivity. The total radioactivity of the processed samples was calculated by using radioactivity of an aliquot measured by LSC, aliquot weights, and supernatant weights. Supernatant was evaporated to near-dryness using a SpeedVac concentrator and reconstituted with 300 µl water: acetonitrile (2:1, v/v). The samples were sonicated, mixed, and analyzed by liquid chromatography/mass spectrometry (LC-MS). Radioactivity was analyzed by scintillation counting. The extensively washed protein pellets were analyzed by LSC after oxidizer treatment.

In vivo sample analysis was performed on an HPLC system consisting of Dionex Ultimate 3000 RS pumps and a Diode Array Detector coupled with either a Lumos Orbitrap or a Q Exactive Plus mass spectrometer (Thermo Fisher

Scientific, San Jose, CA). Liquid chromatography was performed with a Polaris C18-A column (4.6 × 150 mm, 3 µm; Agilent Technologies, Santa Clara, CA) with mobile phases 10 mM ammonium acetate in water with 0.1% formic acid (mobile phase A) and acetonitrile (mobile phase B). The flow rate was 1 ml/min with 10:1 post column split. The HPLC gradient was as follows: initial hold at 5% B for 2 minutes, ramp to 40% B at 15 minutes, hold at 40% B until 28 minutes, ramp to 50% B at 28.1 minutes, hold at 50% B until 50 minutes, and then ramp to 95% B at 54 minutes, hold until 57 minutes, and return to initial equilibrium conditions. Electrospray ionization was used with electrospray voltage set at 4.0 kV and a capillary temperature of 270°C. The full scan mass spectra were obtained at resolving power of 30,000 with accurate mass measurements using external calibration. The corresponding data-dependent tandem mass spectrometry (MS/MS) scans were acquired at a resolving power of 7500 with collision-induced dissociation.

Product Identification in Incubations of GDC-0810, M4, and M6 in Buffer with GSH or Cysteine. Incubations were carried out in 100 mM potassium phosphate buffer (pH 7.4) with either 1 mM cysteine or glutathione. GDC-0810, M6, or M4 at 5 µM (<0.1% DMSO final) were incubated in buffer alone or buffer with 1 mM cysteine or glutathione for 1 hour at 37°C in a shaking water bath (120 rpm). Incubations were quenched on ice. Samples were injected onto a Q Exactive Plus mass spectrometer connected to a Dionex HPLC (Thermo Fisher Scientific). Mass spectrometry full-scan and MS/MS modes were collected. Heated-electrospray ionization ion source was used on the MS in positive mode with a spray voltage of 3500 eV and probe heater temperature of 425°C. Higher energy collisional dissociation energy used for MS² was 25–45. The LC separation used a phenylhexyl 100 × 2.1 mm, 1.7-µ column (Phenomenex, Torrance, CA) with a 0.4 ml/min flow rate of water with 0.1% formic acid (mobile phase A) and acetonitrile with 0.1% formic acid (mobile phase B). The gradient used was as follows: initial hold at 2% B for 1 minute, ramp to 95% B at 12.5 minutes, hold until 13 minutes, and return to initial equilibrium conditions.

Product Identification in Incubations of GDC-0810, M4, and M6 in Liver Microsomes with NADPH and GSH. GDC-0810, M6, or M4 (5 µM) were

TABLE 1

Exposure of radioactivity (nanograms per gram equivalent of [^{14}C]GDC-0810) of [^{14}C]GDC-0810 and its identified metabolites in pooled plasma samples from intact female monkeys after a single oral dose of 50 mg/kg

Metabolites were numbered according to retention time except for parent compound (GDC-0810).

Analyte	Exposure (ng/g equivalent) per Time Point (h)					
	3	6	12	24	48	72
GDC-0810	1151	3332	339	179	22	16
M1 ^a	D	D	D	D	ND	ND
M3	D	32	515	56	22	32
M5 ^b	D	D	176 ^c	D	D	ND
M11 ^b	D	D	D	D	ND	ND
M6	2303	4794	176	100	76	32
M7	D	D	D	D	D	ND
Nanograms equivalents per gram characterized	3454	9206	1206	335	120	80
Nanograms equivalents per gram in sample	4860	12,900	2840	806	515	380
Sample extraction recovery (%)	82	75.9	51.9	45.7	38	25.4

D, detected only by mass spectrometry; ND, not detected.

^aM1 was a di-glucuronide and M3 was oxidative di-glucuronide (see Supplemental Fig. 1).

^bM5 coeluted with M11 in plasma.

^cValue represents total of M5 and M11.

incubated in human liver microsomes or cynomolgus monkey liver microsomes (0.5 mg/ml) in 100 mM potassium phosphate buffer (pH7.4) and GSH (5 mM) in the presence or absence of NADPH (1 mM). After the reaction was complete within an hour, the mixture was quenched with three volumes of cold acetonitrile: methanol (9:1) and chilled on ice for 30 minutes. The resulting suspension was centrifuged at 3000g for 30 minutes, and supernatant was concentrated under nitrogen. Samples were analyzed on a Thermo Fusion Lumos mass spectrometer (Thermo Fisher Scientific) coupled with a Kinetex C18-XB 100 $\times$ 2.1 mm, 1.7- μm , 100 $\text{\AA}$ column (Phenomenex). LC mobile phase containing 0.1% formic acid in water (A) versus 0.1% formic acid in acetonitrile (B) was used, and the gradient was as follows: initial hold at 5% B for 3 minutes, ramp to 50% B at 15 minutes, ramp to 95% B at 18 minutes, hold for 4 minutes, and return to initial equilibrium conditions.

Cysteine Reactivity in Incubations of GDC-0810, M4, and M6 in Buffer with Cysteine. GDC-0810, M6, and M4 at 1 μM were incubated with 5 mM cysteine in 100 μl of 100 mM phosphate buffer, pH 7.4, for 0, 30, 60, 90, 120, 150, and 180 minutes at 37°C in Eppendorf tubes with 800 rpm shaking. The samples were analyzed by Sciex TripleQ 5500 LC-MS/MS analysis with multiple-reaction monitoring in positive mode. The samples were quenched with 100 μl of cold acetonitrile containing two internal standards (pre: diphenhydramine, and post: propanolol) and injected (10 μl) at real time. The percent remaining of parent was plotted against time. Slope and $t_{1/2}$ were calculated by GraphPad. Acrylamide-containing neratinib and acalabutinib were included as positive controls for the cysteine reactivity determination.

Metabolite Formation of GDC-0810 in Hepatocytes of Human and Monkey. GDC-0810 at 1 μM was incubated with cryopreserved hepatocytes of human and monkey (0.5 million cells/ml) for 3 hours at 37°C in Dulbecco's modified Eagle's medium buffer. The samples were analyzed by LC-MS/MS analysis on QE plus (Thermo Fisher Scientific). LC mobile phase containing 0.1% formic acid in water versus acetonitrile was used as a gradient on a Kinetex C18 1.7- μm , 100 $\text{\AA}$, 100 $\times$ 2.1 mm column (Phenomenex). Full scan and MS/MS scans in positive mode were collected to detect metabolites of glucuronide (m/z 623.1591), adducts of CoA (m/z 1196.2317), AMP (m/z 776.1796), conjugates of taurine (m/z 554.1311) or glycine (m/z 504.1485), and conjugates of the acyl glucuronide with GSH (m/z 930.2429) or cysteine (m/z 744.1788).

Quantum Mechanical Methods. The model compounds cinnamic acid and its O-glucuronide were used for this analysis. Conformers of the O-glucuronide (B) were generated using the "conformer distribution" functionality within Spartan '18 (Wavefunction, Inc., Irvine, CA) with the software's implementation of MMFF (Halgren, 1996a,b,c,d; Halgren and Nachbar, 1996). Conformers were then exported, and geometry optimizations were run with Gaussian09 (Revision E.01; Gaussian Inc., Wallingford, CT, 2013; see Supplemental Information for full reference). Initial geometry optimizations of the conformers of B were run at the HF (Roothaan, 1951; Cossi et al., 2003) level with the 6-31G(d) basis set and implicit CPCM (Barone and Cossi, 1988) solvent model for water. These initial geometry optimizations were used to identify the lowest energy conformers for

further optimization at higher levels of theory. The top two conformers of B and the sole conformer of cinnamic acid (A) were then optimized at the M06-2X (Zhao and Truhlar, 2008) level of theory with the 6-31+G(d) basis set and implicit CPCM solvent model for water. The lowest energy conformers of B and A were then subjected to a single point calculation within Spartan '18 (Q-Chem implementation within Spartan '18; Shao et al., 2015) at the M06-2X level of theory with the 6-311+G(2df,2p) basis set and the implicit CPCM solvent model for water (Supplemental Table 2). A three-dimensional rendering of the lowest unoccupied molecular orbital (LUMO) of B was made within Spartan '18.

Results

Metabolic Pathways of GDC-0810 in Monkeys. The major clearance pathway of GDC-0810 in monkeys was through acyl glucuronidation, with M6 as the major metabolite in plasma, bile, and urine (Supplemental Figs. 1 and 2; Table 2). Other metabolites identified in monkey plasma and excreta include di-glucuronide (M1), oxidative di-glucuronide (M3), di-glucuronide (M4), oxidative glucuronide (M5), oxidative metabolite (M7), and cysteine adduct of M6 (M11). The radioactivity extraction recoveries from monkey plasma decreased from 82% at 3 hours to less than 50% at 24 hours and to a lower recovery at later time points postdose (Table 1). The extraction recovery was approximately 75% from 0- to 168-hour fecal samples. Subsequent oxidation of the protein precipitate pellet showed that the unextractable radioactivity was associated with the pellet from plasma samples. The mass balance and metabolites identified in plasma, urine, bile, and feces of monkeys after oral administration of [^{14}C]GDC-0810 are included in the Supplemental Information. The radioactive metabolite profiles of GDC-0810 in monkey urine, bile, and feces are also shown in the Supplemental Fig. 2 and Supplemental Table 1.

There were two metabolites (M6 and M11) in plasma extract that were of particular interest. The radioactive peak of M6 (m/z 623) was a major circulating metabolite (Fig. 2). The MS/MS spectra showed product ions at m/z 605 (loss of H_2O), 447 (loss of anhydroglucuronic acid), 299 (loss of phenyl propenoic acid), and 311 (loss of indazole and H_2O). The elemental composition of M6 was confirmed using accurate mass analysis. The fragmentation pattern of M6 observed in study samples was similar to that of the standard (Supplemental Fig. 3).

The radioactive peak of M11 (m/z 744) was observed in monkey plasma as well as in urine and bile. The mass spectra showed product ions at m/z 726 (loss of H_2O), 623 (loss of cysteine), 568 (loss of anhydroglucuronic acid), and 447 (loss of anhydroglucuronic acid and cysteine). The elemental composition of M11 was confirmed by accurate

TABLE 2
Molecular ion and major product ions observed for GDC-0810 and its metabolites

Analyte	RT(min) ^a	Observed [M + H] (m/z)	Theoretical [M + H] (m/z)	Mass Accuracy (ppm)	Chemical Formula	Matrix ^b	Major Fragment Ions (m/z)
GDC-0810	45.96	447.1270	447.1270	0.0	C26H21ClFN2O2 ⁺	p, u, b, f	429.1179, 299.0759, 284.0523, 263.0992, 311.0647, 169.0764, 143.0060
M1 ^c	17.29	799.1917	799.1912	-0.6	C38H37ClFN2O14 ⁺	p, u	623.1625, 447.1294, 299.0770
M3 ^c	17.84	815.1860	815.1861	0.1	C38H37ClFN2O15 ⁺	p, u, b	797.1779, 639.1554, 621.1442, 463.1226, 445.1122, 315.0706, 309.0478
M4	19.63	623.1592	623.1591	-0.2	C32H29ClFN2O8 ⁺	p, u, b	605.1494, 447.1287, 311.0655, 299.0760
M5	17.14	639.1540	639.1540	-0.2	C32H29ClFN2O9 ⁺	p, u, b	505.1396, 463.1237, 446.1181, 315.0710, 309.0478
M6	30.86	623.1590	623.1591	0.2	C32H29ClFN2O8 ⁺	p, u, b	605.1500, 447.1286, 311.0645, 299.0759, 263.0961
M7	39.17	463.1216	463.1219	0.6	C26H21ClFN2O3 ⁺	b	446.1206, 411.1521, 315.0708, 311.0648, 185.0713, 143.0059
M11	18.29	744.1790	744.1788	-0.3	C35H36ClFN3O10S ⁺	p, u, b	726.1737, 623.1620, 568.1501, 447.1300, 403.1398
M13	7.11	465.6254 ^d	465.6251	-0.6	C27H29F3N5O8 ⁺	In vitro	447.1266, 405.1169, 299.0750, 203.0624, 162.0220, 130.0501
M14	7.48	377.6089 ^d	377.6090	0.3	C36H38ClFN5O8S ⁺	In vitro	447.1272, 405.1167, 340.0946, 299.0743, 203.0624, 162.0217, 130.0501, 84.0449

b, bile; f, feces; [M + H]⁺, protonated molecular ion; p, plasma; RT, retention time (min); u, urine.

^aLC-MS retention times are from metabolite profiling of matrix that has the most abundant metabolites. Some retention time shift was observed for different matrices.

^bSample matrix showing radioactive peak (excluding buffer).

^cM1 was a di-glucuronide and M3 was oxidative di-glucuronide.

^dZ = 2 doubly charged.

mass analysis (Fig. 3). Based on these fragmentation data, M11 was tentatively assigned as the cysteine adduct of GDC-0810 glucuronide M6. The assignment of M11 was further confirmed by in vitro incubation of M6 with cysteine in buffer.

Adduct Formation of GDC-0810, M6, and M4 In Vitro. GDC-0810, along with its glucuronide metabolites (M4 and M6), were individually incubated in phosphate buffer in the presence of cysteine or GSH to examine the potential for chemical reactivity. After analysis via LC-MS, no GSH (+307 Da) or cysteine (+121 Da) adducts were detected for GDC-0810 (Fig. 4A). Similar results were observed after incubation of the *N*-glucuronide metabolite M4, with no GSH adduct and a very low abundance of cysteine adduct observed (>1000-fold less). Conversely, the GSH conjugates of the glucuronide metabolite M13 (M+307 of M6) were relatively abundant in the GSH incubation (Fig. 4C). This was also observed with the corresponding cysteine conjugate (M+121 of M6, M11) in the cysteine incubation (Fig. 4B). Acyl-migration for M6 is likely responsible for the peaks with the same *m/z* or its cysteine conjugate in the M6 incubations. These results suggest that the chemical reactivity of the acyl *O*-glucuronide M6 is much higher than that of GDC-0810 or its *N*-glucuronide metabolite M4. For acyl glucuronide M6, time-dependent acyl migration was observed. Two cysteine conjugate peaks observed for M11 are likely a result of acyl-migration isomers. Only one peak observed in in vivo plasma could be a result of less acyl migration in the plasma or a coelution under the LC method used. The broader M13 peak formed from the reaction of M6 with GSH could result from coeluting peaks.

The structural proposal of M13 (*m/z* 930) is supported by LC-MS/MS fragmentation data (Fig. 5). Fragmentation was conducted by collision-induced dissociation and higher energy collisional dissociation to characterize GSH conjugates. Fragment ions of *m/z* 801 and *m/z* 625 resulted from neutral loss of γ -glutamate (129 Da) and subsequent neutral loss of glucuronide moiety (176 Da), respectively. Ions *m/z* 754 and *m/z* 447 result from neutral losses of glucuronide (176 Da) and glucuronide + GSH moieties (176 + 307 Da). Fragment ions *m/z* 405 and *m/z* 299 suggested the addition of GSH on the allyl group, supporting the stability of GDC-0810 aromatic ring system.

The Michael addition of cysteine to M6 to form M11 was efficient with a half-life of 58 minutes. For comparison, k_{react} (in $\text{M}^{-1}\text{s}^{-1}$; $t_{1/2}$ in minutes) were 0.043 (26.8) and 0.0077 (149.8) for the two acrylamide-containing drugs neratinib and acalabrutinib, respectively.

M13 formed in situ was subject to glucuronidase treatment, and the GSH adduct of GDC-0810 (M14) was identified as a product of the enzymatic incubation (Supplemental Fig. 4). The results suggested that the Michael addition product was stable even after removal of the glucuronic acid group and provides further evidence that the formation of M13 is from M6 rather than from GDC-0810. The M14 (*m/z* 754) LC-MS/MS fragmentation pattern also suggests the addition of GSH on the allyl group because of fragment ions *m/z* 405 and *m/z* 299 (Table 1). Double-charged fragment ion *m/z* 340 resulted from neutral loss of glycine (75 Da), and ion *m/z* 447 resulted from neutral loss of GSH moiety (307 Da).

GDC-0810 and its glucuronide metabolites (M4 and M6) were also incubated in monkey or human liver microsomes in the presence NADPH and GSH to examine the potential for P450-mediated bioactivation and reactive metabolite formation. LC-MS showed that no GSH (M+307 or 305) adducts were detected for GDC-0810 or M4 (Fig. 6, A and C). For M6, although the GSH conjugate M13 (M+307) was the major product, there were no adducts resulting from oxidative metabolites [M+16(32)+305(307)] (Fig. 6B) from these incubations.

Metabolite Formation of GDC-0810 in Hepatocytes. Full scan and MS/MS scan analysis showed that M6 and M4 were prominent metabolites in incubations of GDC-0810 with human and monkey

Fig. 2. Radioactivity profiles of plasma extract of monkeys after oral administration of [^{14}C]GDC-0810.

hepatocytes. No CoA or AMP adduct was identified. Taurine or glycine adducts and cysteine or GSH conjugates of M6 were also not identified.

Quantum Mechanical Calculations. Acids, in general, are weaker electrophiles than esters. This is due to the significant electron density of the carboxylate, which hinders the addition of an electron-rich nucleophile. The weaker reactivity of acids applies to both

1,2-addition to the carbonyl and Michael addition to a corresponding alkene. Density functional theory computations were performed to quantitatively characterize the electrophilicity of the two compounds of interest. Models of the GDC-0810 (cinnamic acid, A) and M6 (B) were used for this analysis (Fig. 7). As GDC-0810 and M6 are identical aside from the respective carbonyl functionalities, the

Fig. 3. Product ions (MS/MS spectra) of M11. ESI, electrospray ionization; cid, collision-induced dissociation; F, scan filter; FTMS, Fourier transform mass spectrometry; NL, normalized level to the average base peak; RT, retention time (minutes).

difference in reactivity can primarily be attributed to the difference in the acid and ester moieties. Thus, the truncated models provide sufficient and easy insight into the inherent reactivity. Figure 7 also displays the corresponding computed energy level of the LUMO and electrophilicity index (ω) (Parr et al., 1999; Chattaraj et al., 2006; Domingo et al., 2016) of each model compound. As the LUMO energy is lowered, the orbital becomes more receptive toward nucleophilic addition. Additionally, ω is another measure of electrophilicity, in which larger indices are indicative of stronger electrophiles (Parr et al., 1999; Chattaraj et al., 2006; Domingo et al., 2016). As shown in Fig. 7, B has both a lower lying LUMO and a larger electrophilicity index, indicating that it is a better electrophile than A. These calculated results are consistent with the experimental observations. Michael acceptors, such as the activated α,β -unsaturated ester B, are inherently reactive at the β position. This inherent reactivity is also visible in the LUMO depiction of Fig. 7, in which the lobe of the LUMO on the β -carbon (4-position) appears visibly larger than that of the carbonyl carbon (2-position) and supports formation of GSH- or cysteine-adduct products at this site. The *in silico* results quantitatively explain how acyl glucuronidation enhances the reactivity of the original α,β -unsaturated acid and therefore allows for covalent binding to proteins.

Discussion

To investigate the mechanism of protein covalent binding of the metabolites of GDC-0810, several *in vitro* experiments were conducted similar to those outlined in Zhang et al. (2012).

A direct conjugate of GDC-0810 with GSH or cysteine was not observed in *in vitro* incubations, yet direct conjugates were quickly formed in incubations with M6 to form M13. The fragmentation analysis of M13, the GSH adduct of M6, showed that GSH added to the β -carbon and not to the glucuronic acid moiety through a potential Schiff base (Fig. 1). The Michael addition of the cysteine to M6 was very efficient with a half-life of less than 60 minutes. There was no glucuronic acid replacement product by GSH or cysteine from these incubations. Direct replacement of acyl glucuronide by cysteine thiol, followed by *S*- to *N*-acyl rearrangement to form a more stable conjugate, has previously been reported (Harada et al., 2019). This produces an isomer with the same molecular ion in mass spectral analysis. We did not detect a similar displacement product during this analysis of GDC-0810. This is explained by the inherent reactivity differences between the compounds studied by Harada et al. (2019) and the Michael acceptor M6 found here. The acids in Harada et al. (2019) are aliphatic or aromatic and do not contain reactive conjugated alkenes; GDC-0810 contains an α,β -unsaturated carbonyl, which is activated by converting the acid

Fig. 4. LC-MS product profiles of incubations of GDC-0810 (A), M4 (B), and M6 (C) in buffer in the presence of GSH or cysteine.

(GDC-0810) to the ester (M6). The glutathione S-transferase catalyzed addition of GSH to electrophilic substrates is a known source of GSH conjugation to many covalent modifier drugs and reactive metabolites (Ploemen, et al., 1994; Okada et al., 2011). However, M13 was not an identified *in vivo* metabolite in monkeys. In addition, a GSH conjugate

of GDC-0810 was not formed in incubations of monkey and human liver microsomes with GDC-0810 in the presence of GSH. Our study exemplifies how the identity of the electrophile, a Michael acceptor in this example, can dictate the reactivity with biologically active thiols. Quantum mechanical calculations were able to quantify the inherent

Fig. 5. Product ions (MS/MS spectra) of M13.

reactivity differences between α,β -unsaturated functional groups and illustrate how computations can complement our understanding to better predict the susceptibility of electrophilic moieties to nucleophiles such as GSH. This should be considered when elucidating the metabolic profile of compounds.

The GDC-0810 scaffold contains two extensively conjugated alkene moieties generally considered to be susceptible to P450-mediated epoxidation, forming reactive intermediates. These reactive intermediates could be susceptible to further reactions such as hydrolytic ring opening and alkylation. We were intrigued to find that after incubation with human or monkey liver microsomes, no oxidative metabolites of GDC-0810 (M+16) were detected. Additionally, when M4, M6, and GDC-0810 were incubated with liver microsomes fortified with NADPH and GSH, GSH conjugation to oxidized metabolites (M+323) was not observed. M3, an oxidative metabolite of the di-glucuronide M1, was a prominent circulating metabolite in monkeys in vivo, along with M6, but the potential reactivity of M3 could not be assessed because of a lack of a metabolite standard. These results suggest that the potential for P450-mediated bioactivation for GDC-0810 is very low in monkeys or humans. Acyl glucuronidation of gemfibrozil and clopidogrel led to CYP2C8-mediated bioactivation. This resulted in significant clinical drug-drug interactions with those drugs metabolized by CYP2C8 (Ogilvie et al., 2006; Ma et al., 2017; Itkonen et al., 2019). P450 inactivation was not reported in the case of GDC-0810. It should also be

mentioned that toxicity findings associated with GDC-0810 bioactivation have not been reported.

From the incubations in liver microsomes fortified with NADPH and GSH, we observed the GSH conjugate of glucuronide metabolite M13 (M+307 of M6) instead. This is consistent with the previous experiment in buffer incubations with M6. These observations suggest that GDC-0810 is explicitly bioactivated via glucuronidation. No GSH adducts were identified from the trapping incubations with GDC-0810 or M4.

The mechanism of bioactivation discussed within this manuscript is distinct from previously reported examples. Carboxylic acid-containing compounds can form acyl CoA conjugates through acyl adenylate intermediate (AMP). The acyl CoA conjugates can be more reactive than the corresponding acyl glucuronides and can further react to form glycine or taurine conjugates (Grillo et al., 2003, 2012; Darnell et al., 2015; Lassila et al., 2015). Here, no acyl adenylate intermediate metabolites were observed in incubations of GDC-0810 in hepatocytes. Although the Michael addition of a thiol to the acyl glucuronide of GDC-0810 was efficient, which can explain its protein binding potential, the possibility of forming acyl CoA thioester conjugates as a contributing factor of covalent binding of GDC-0810 cannot be ruled out. Identification of in vitro acyl CoA conjugates would be complicated by rapid degradation in the hepatocyte incubations, and our in vitro methods were not optimized for detection.

Fig. 6. LC-MS product profiles of incubations of GDC-0810 (A), M4 (B), and M6 (C) in monkey liver microsomes in the presence of NADPH and GSH or cysteine.

Another covalent binding mechanism appeared to correlate, in general, with the chemical stability of the acyl glucuronides (Sawamura et al., 2010; Zhang et al., 2011; Zhong et al., 2015). M6 is very stable in buffer with a half-life of greater than 8 hours. In comparison, diclofenac acyl glucuronide has a half-life of less than 1 hour under similar conditions (Zhang et al., 2011). This incredible stability indicates that although M6 is converted to a great electrophile for protein conjugation, it is not activated to undergo degradation or other metabolic processes.

Bioactivation by glucuronidation has only been previously described to result in transacylation or glycation via Amadori rearrangement (Ding et al., 1993; Skonberg et al., 2008; Miyashita et al., 2014; Harada et al., 2019). No GSH addition to the glucuronic acid moiety was observed, although acyl migration isomers were detected in these in vitro incubations. Collectively, our results suggested that bioactivation of α,β -unsaturated acids was via glucuronidation and thus led to its covalent binding to proteins via Michael addition. Acyl glucuronidation

Fig. 7. (Left) Models of acid (A) and glucuronide (B) with corresponding LUMO energies and electrophilicity index (ω); (Right) depiction of the LUMO of B.

of GDC-0810 increased the inherent reactivity, making the β -position of the double bond more reactive toward nucleophiles.

Tandem bioactivation–protein conjugation with the acyl glucuronidation of α,β -unsaturated acids represents a novel bioactivation mechanism. There are limited examples that demonstrate the chemical modifications that occur after non-P450 mediated or P450-mediated bioactivation and the impact on covalent binding to proteins (Gan et al., 2016). Covalent binding of acetaminophen to proteins occurs through conjugation of a cysteine thiol to its quinone imine intermediate (Leeming et al., 2015). MaxiPost is reported to form irreversible covalent binding with plasma proteins, mostly serum albumins, in humans, dogs, and rats through Michael addition of the ε -amino group of an albumin lysine to a proposed quinone methide intermediate (Zhang et al., 2005). A number of covalent inhibitors (e.g., neratinib, osimertinib, and ibrutinib) also have shown certain degrees of endogenous protein adduct formation. Neratinib was found to form covalent adducts on Lys-190 of human serum albumin but did not react to the free thiol of cysteine in albumin (Wang et al., 2010). GDC-0810 was quantitatively recovered from 24-hour incubations in human or monkey plasma, supporting that there was no direct reaction between the compound and plasma proteins. Our study demonstrates a new mode of tandem bioactivation–protein conjugation with the acyl glucuronidation of GDC-0810. It is interesting to note that AZD9496, also a target protein degrader, contains an α,β -unsaturated carboxylic acid as well (De Savi et al., 2015; Puyang et al., 2018). Given our studies within this paper, one would expect that a similar bioactivation–protein conjugation pathway is feasible for this candidate. Several reports of α,β -unsaturated carboxylic acid glucuronide metabolites exist in literature, but none mention Michael addition reactions as a possible bioactivation mechanism. Primary considerations of these reports on the risk of covalent binding were associated with acyl glucuronide rearrangements (Sass et al., 1995; Kaul and Olson, 1998; Nakazawa et al., 2003; Kenny et al., 2005; Piazzon et al., 2012).

α,β -Unsaturated amides are excellent Michael acceptors, and a number of covalent inhibitor drugs (e.g., neratinib, osimertinib, and ibrutinib) are designed with acrylamide moieties to react with free thiols of cysteine residues in target proteins. α,β -Unsaturated esters have also been explored as potential warheads to react with free thiols in target proteins (Gehring and Laufer, 2019). It is not known if M6 could covalently bind to ER α protein, leading to degradation of the target. Additional studies would be needed to evaluate if an α,β -unsaturated carboxylic acid can serve as the prodrug strategy, utilizing our identified bioactivation mechanism, to activate a compound for covalent binding to the target receptor.

In summary, acyl glucuronidation of the α,β -unsaturated carboxylic acid in GDC-0810 activates the conjugated alkene toward nucleophilic addition by GSH or other reactive thiols. This is the first example of bioactivation of α,β -unsaturated carboxylic acid compounds leading to covalent binding to proteins.

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Authorship Contributions

Participated in research design: Mulder, Khojasteh, D. Zhang.
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Wrote or contributed to writing of the manuscript: Mulder, Johnson, Grandner, Khojasteh, D. Zhang.

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