Identification of Novel Pathways of Osimertinib Disposition and Potential Implications for the Outcome of Lung Cancer Therapy

A. Kenneth MacLeod, De Lin, Jeffrey T.–J. Huang, Lesley A. McLaughlin, Colin J. Henderson, and C. Roland Wolf

Abstract

Purpose: Osimertinib is a third-generation inhibitor of the epidermal growth factor receptor used in treatment of non–small cell lung cancer. A full understanding of its disposition and capacity for interaction with other medications will facilitate its effective use as a single agent and in combination therapy.

Experimental Design: Recombinant cytochrome P450s and liver microsomal preparations were used to identify novel pathways of osimertinib metabolism in vitro. A panel of knockout and mouse lines humanized for pathways of drug disposition were used to establish the relevance of these pathways in vivo.

Results: Although some osimertinib metabolites were similar in mouse and human liver samples there were several significant differences, in particular a marked species difference in the P450s involved. The murine Cyp2d gene cluster played a predominant role in mouse, whereas CYP3A4 was the major human enzyme responsible for osimertinib metabolism. Induction of this enzyme in CYP3A4 humanized mice substantially decreased circulating osimertinib exposure. Importantly, we discovered a further novel pathway of osimertinib disposition involving CYP1A1. Modulation of CYP1A1/CYP1A2 levels markedly reduced parent drug concentrations, significantly altering pharmacokinetics (PK) in humanized mice in vivo.

Conclusions: We demonstrate that a P450 enzyme expressed in smokers’ lungs and lung tumors has the capacity to metabolise osimertinib. This could be a significant factor in defining the outcome of osimertinib treatment. This work also illustrates how P450-humanized mice can be used to identify and mitigate species differences in drug metabolism and thereby model the in vivo effect of critical metabolic pathways on anti-tumor response. Clin Cancer Res; 24(9); 2138–47. ©2018 AACR.

Introduction

Lung cancer is both the most common cancer, with an estimated 1.8 million new cases in 2012, and the most common cause of mortality from cancer, responsible for 1.5 million deaths in 2012 (1). Non–small cell lung cancer (NSCLC) accounts for 80% to 90% of all cases. A distinct subset of NSCLC—between 8% and 30%, depending on ethnicity (2)—possess activating mutations in the kinase domain of EGFR (“EGFRm”), which confer sensitivity to therapy involving “first generation” EGFR inhibitors, erlotinib and gefitinib (3, 4). As with many tyrosine kinase inhibitors (TKIs), however drug resistance emerges rapidly, with disease progression typically occurring after 9 to 14 months (5). The predominant mechanism of acquired resistance involves a substitution of methionine for threonine in codon 790 (T790M) in EGFR, arising through point mutation in exon 20. This mutation hinders drug binding (6) while also increasing the affinity for ATP (7), and occurs in 50% to 60% of patients who develop resistance to erlotinib or gefitinib (8). Several second-generation EGFR inhibitors, which target both EGFRm and T790M, have undergone clinical trial, with afatinib now approved for first-line therapy in EGFR mutation-positive NSCLC. However, the efficacy of these agents against the T790M mutant protein is disputed, as T790M emerges at a similar frequency in EGFRm+ patients treated with afatinib (9). Hence a third generation of EGFR inhibitors, designed to target both EGFRm and T790M, while sparing wild-type EGFR, is emerging.

Osimertinib is a mono-anilino-pyrimidine which covalently and irreversibly binds to cysteine 797 in the ATP binding site of EGFR. Osimertinib exhibited 200 times greater potency towards EGFRm/T790M than EGFRWT (5). Compared with first and second-generation inhibitors, cell line studies demonstrated a similar activity against EGFRm, increased activity against EGFRT790M, and an increased selectivity margin against EGFRWT (5). On phase I and II clinical trial data, osimertinib was granted accelerated approval by the FDA and the EMA for treatment of metastatic EGFR T790M–positive NSCLC which had progressed on or after TKI therapy. It was subsequently reported that the Phase III trials of osimertinib met its primary endpoint of increased progression-free survival (PFS), compared to standard platinum-doublet chemotherapy, with evaluation of overall survival ongoing (10). Response rates to therapy are approximately 70%—of which 3% are complete and 67% partial—with a median response duration of 11.4 months as, again, resistance is acquired rapidly (11). Several mechanisms of resistance have been identified, most of which are shared with first-generation EGFR inhibitors, such as mutation...
Translational Relevance

Globally, there are approximately 1.5 million new cases of non–small cell lung cancer (NSCLC) each year. Dependent on ethnicity, between 8% and 30% of these contain mutations in the epidermal growth factor receptor (EGFR), which confer sensitivity to therapy with inhibitors of this oncoprotein. Acquired resistance to first and second-generation EGFR inhibitors usually involves a further EGFR mutation, T790M. Osimertinib (AZD9291) is the most effective treatment in these latter cases and, due to its low toxicity and high level of brain penetration, is currently being explored as a first-line therapy for metastatic disease. Osimertinib is a cytochrome P450 substrate. These enzymes can define both systemic exposure and intratumoral drug concentrations and can therefore be major determinants in the outcome of cancer therapy. We report a novel pathway of osimertinib disposition which may be of importance in this regard.

Materials and Methods

Chemicals and reagents

High/low P450 activity HLM preparations from individual donors were purchased from BD Gentest. Pooled human liver microsomes (150 donors) were purchased from Thermo Fisher. Human and murine microsomal preparations, and knockout and hCYP3A4/3A7 mice has been described previously (19). All incubations were performed in 100 mmol/L potassium phosphate buffer, pH 7.4, containing 3.3 mmol/L MgCl₂ at 37°C and 300 rpm in a thermomixer. Further details are available in Supplementary data.

Western blotting

Microsomal fractions were adjusted to 1 mg/mL in LDS sample buffer (Life Technologies). Primary antibodies used for immunoblotting were anti-CYP1A1 (AB1258; Merck Millipore) and anti-GRP78 (ab21685; Abcam).

Cell line studies

The A549_HO1 and H1299_HO1 cell lines will be described in detail in a future publication. Briefly, parental A549 and the CYP2D6 promoter, along with all exons, introns, and 5’ and 3’ untranslated regions, into the murine Cyp2d locus, from which all nine functional murine Cyp2d genes have been deleted (20). The hPXR/hCAR/hCYP3A4/3A7 line carries a large genomic insertion of human CYP3A4 and CYP3A7 in place of the Cyp3a locus, from which seven of the eight murine Cyp3a genes have been deleted (21). CYP3A4 is expressed at a low basal level in this line but can be upregulated following ligand-activated nuclear translocation of the transcription factor, PXR, by rifampicin (RIF). To generate the hPXR/hCAR/3aKO line, we crossed hPXR/hCAR with Cyp3aKO animals (21). A manuscript describing the generation and characterization of the hCYP1A1/1A2 and Cyp1a1/1a2KO lines is in preparation. All animals were maintained under standard animal house conditions, with free access to food (RM1 diet, Special Diet Services) and water, and a 12 h light/dark cycle. All animal work was carried out in accordance with the Animal Scientific Procedures Act (1986) and after local ethical review.

In vitro studies

Microsomal protein fractions were prepared as described previously (23). All incubations were performed in 100 mmol/L potassium phosphate buffer, pH 7.4, containing 3.3 mmol/L MgCl₂ at 37°C and 300 rpm in a thermomixer. Further details are available in Supplementary data.

LC-MS/MS: multiple reaction monitoring analysis

Analysis of in vitro incubation and in vivo blood PK samples was carried out by UHPLC/MS-MS using a Waters Acquity UPLC (Micromass) and Micromass Quattro Premier mass spectrometer (Micromass) with Electrospray detection. Further details are available in Supplementary data.

LC-MS/MS: MS² analysis

The LC-MS/MS system consisted of a Waters Alliance 2690 HPLC system (Waters) and a LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific) with an electrospray ionization (ESI) interface. Further details are available in Supplementary data.

Western blotting

Microsomal fractions were adjusted to 1 mg/mL in LDS sample buffer (Life Technologies). Primary antibodies used for immunoblotting were anti-CYP1A1 (AB1258; Merck Millipore) and anti-GRP78 (ab21685; Abcam).

Cell line studies

The A549_HO1 and H1299_HO1 cell lines will be described in detail in a future publication. Briefly, parental A549 and
NCl-H1299 were obtained from the ATCC. Genes for firefly luciferase and beta-galactosidase were introduced C-terminal to the HO1 gene in both lines using the transcription activator-like effector nuclease (TALEN) genome editing system. These reporter genes are separated from each other, and from HO1, by the 2A sequence derived from foot-and-mouth disease virus, leading to the production of a tricistronic mRNA and thereby allowing efficient production of all three proteins from the HO1 promoter. The AReC32 cell line has been described previously (24). All cell lines were cultured in DMEM (Life Technologies) containing 10% FBS and verified as mycoplasma-free (MycoAlert). For induction studies, cells were exposed to osimertinib in complete medium for 24 h and luciferase activity measured using a commercially available kit (Promega).

Data analysis
Spearman’s rank correlation coefficients were calculated in Microsoft Excel (Microsoft). PK parameters of in vivo data were calculated with a simple non-compartmental model using the PK Functions package in Microsoft Excel and P values were calculated using an unpaired, two-tailed t test. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001.

Results
In vitro metabolism of osimertinib: similarities and differences between human and mouse
To determine the suitability of the mouse as a model system to investigate pathways of osimertinib disposition in vivo, we first compared the metabolism of this drug in mouse and human liver microsomal fractions in vitro. Analysis of samples from incubations with HLM and wild-type MLM identified two demethylated (designated DM-1 and DM-2) and five hydroxylated (designated OH-1 to OH-5) metabolites in each species (Fig. 1A and B). The demethyl-osimertinib profile was closely-matched between species (Fig. 1C). However, the hydroxylated forms were produced at very different ratios, and two were produced in much greater quantities by MLM (Fig. 1D and E). Manual fragmentography confirmed that the seven metabolites produced by MLM were the same as those produced by HLM, and that DM-1 and DM-2 are the previously reported clinically-important AZ5104 (M6) and AZ7550 (M3) metabolites, respectively (Supplementary Fig. S1; refs. 25, 26). The structure of osimertinib and sites of modification are shown in Fig. 1F. In previous reports, only three mono-hydroxylated metabolites have been identified (25, 26). OH-4 corresponds to M1, but structural detail for the other two previously identified metabolites—designated M4 and M7—are unavailable for comparison with our data (25, 26).

To further clarify the human cytochrome P450s involved in osimertinib metabolism we carried out incubations with a panel of recombinant P450 enzymes. CYP3A4 and CYP1A1 were the most active in generating DM-1, exhibiting approximately equivalent activity (Fig. 2A). CYP3A4 played a major role in generating DM-2, with minor roles for CYP1A1 and CYP2C8 (Fig. 2B). Interestingly, CYP1A1 had by far the highest activity in forming the OH-1, OH-3, and OH-5 metabolites (Fig. 2C, E and G), whereas CYP1A2 and, to a lesser extent, CYP3A4, CYP1A1, and CYP2C8 produced OH-2 (Fig. 2D). CYP3A4 was most active in the generation of the 4-OH metabolite (Fig. 1F).
To establish which cytochrome P450s play a predominant role in the production of these metabolites in human liver we used a panel of HLM from 15 donors, characterized in their activity towards probe substrates for individual P450 isoforms. The formation of DM-1 correlated strongly with that of 6β-hydroxy-testosterone, the marker for CYP3A4, agreeing with the recombinant protein data (Supplementary Table S2). There was also a strong correlation with CYP2A6 activity but we deemed this potentially artifactual as CYP3A4 and CYP2A6 probe activities within the HLM panel correlate (not shown). This relationship has previously been observed in a separate panel of HLM samples (27). Indeed, recombinant CYP2A6 did not produce this metabolite (Fig 2A). It should be noted however that Dickinson and colleagues observed some activity of recombinant CYP2A6 with osimertinib (26). Formation of DM-2 in the HLM panel agreed less well with the recombinant protein data, as correlation with CYP3A4 activity was considerably weaker than with CYP2A6, CYP2B6, and CYP2C8. OH-1 data were consistent as, in the absence of a reference CYP1A1 activity in the HLM panel, CYP3A4 was the next strongest correlation (disregarding CYP2A6). Generation of OH-2 correlated well with CYP3A4 and CYP2C8 activities, consistent with the recombinant data but, in stark contrast, the predicted CYP1A2 correlation was not observed. The reason for this is unclear. Consistent with the recombinant P450 data, the correlation of OH-4 formation with CYP3A4 activity was highest. This analysis did not clearly identify the enzymes involved in the formation of the OH-3 and OH-5 metabolites (Supplementary Table S2).

In addition to the recombinant protein and probe substrate correlation analyses, we further characterized the P450s involved in osimertinib disposition using isoform-specific inhibitors in pooled HLM. In broad agreement with the above work, the generation of DM-1, DM-2, OH-1, and OH-2 were strongly inhibited by the CYP3A4 inhibitor ketoconazole (Supplementary Table S3). Further, in agreement with the correlation analysis, inhibition of CYP1A2 did not alter production of OH-2. Because of the reported low basal level of expression of CYP1A1 in human liver (28), we did not attempt to inhibit this enzyme in HLM.

To further characterize the involvement of CYP3A4 and CYP1A1 in osimertinib metabolism, we determined the apparent kinetic parameters for conversion to DM-1, DM-2, and OH-1 by recombinant forms of these enzymes in vitro. All data were in agreement with the above: (i) both CYP1A1 and CYP3A4 generated DM-1, with the former demonstrating a far lower $K_m$ and the latter demonstrating a higher $V_{max}$; (ii) CYP1A1 was essentially inactive in the formation of DM-2 with CYP3A4 exhibiting significant activity (iii) both enzymes generated OH-1, but CYP1A1 had both a lower $K_m$ and higher $V_{max}$ (Supplementary Table S4).

Osimertinib is an irreversible inhibitor of EGFR containing an acrylamide reactive center, which binds covalently to cysteine 797 in the kinase active site (5). Specificity for this target is enhanced through the formation of two hydrogen bonds between the pyrimidine core of osimertinib and methionine 793 of EGFR. This alpha/beta unsaturated ketone moiety is thiol-reactive (29), a common feature of many activators of the transcription factor nuclear erythroid 2 related factor 2 (NRF2). NRF2 provides a primary defense against reactive electrophiles and has been associated with drug resistance in cancer (30). We therefore tested the ability of osimertinib to activate NRF2-driven luciferase reporters in three stable cell lines; two driven off the endogenous heme oxygenase 1 (HO1) promoter, A549_HO1 and H1299_HO1, for which NRF2 is one of a number of regulators, and one driven off a synthetic promoter containing eight copies of the antioxidant response element (ARE), which is specific for NRF2, AREc32 (24).

Murine Cyp2d are responsible for the species difference in osimertinib metabolism

As described by Cross and colleagues, the half-life of oral clearance of osimertinib is approximately three hours in the mouse (5). However, in early clinical studies, the human value was found to be approximately 50 hours, "longer than would be the case for mice. Therefore, the target species for in vivo studies of osimertinib metabolism would be the humanized, Cyp2d-deficient mouse. In preliminary studies, the humanized mouse with a Cyp2d knock-out background failed to generate metabolites associated with osimertinib metabolism in vivo. To extend these results, we characterized the in vitro metabolism of osimertinib in a panel of recombinant human cytochrome P450s. Osimertinib was incubated with recombinant P450 as described in the Materials and Methods and conversion to (A) DM-1, (B) DM-2, (C) OH-1, (D) OH-2, (E) OH-3, (F) OH-4, and (G) OH-5 forms measured by LC-MS/MS.

Data shown are mean ± SD of triplicate incubations and are representative of three separate experiments.

To establish which cytochrome P450s play a predominant role in the production of these metabolites in human liver we used a panel of HLM from 15 donors, characterized in their activity towards probe substrates for individual P450 isoforms. The formation of DM-1 correlated strongly with that of 6β-hydroxy-testosterone, the marker for CYP3A4, agreeing with the recombinant protein data (Supplementary Table S2). There was also a strong correlation with CYP2A6 activity but we deemed this potentially artifactual as CYP3A4 and CYP2A6 probe activities within the HLM panel correlate (not shown). This relationship has previously been observed in a separate panel of HLM samples (27). Indeed, recombinant CYP2A6 did not produce this metabolite (Fig 2A). It should be noted however that Dickinson and colleagues observed some activity of recombinant CYP2A6 with osimertinib (26). Formation of DM-2 in the HLM panel agreed less well with the recombinant protein data, as correlation with CYP3A4 activity was considerably weaker than with CYP2A6, CYP2B6, and CYP2C8. OH-1 data were consistent as, in the absence of a reference CYP1A1 activity in the HLM panel, CYP3A4 was the next strongest correlation (disregarding CYP2A6). Generation of OH-2 correlated well with CYP3A4 and CYP2C8 activities, consistent with the recombinant data but, in stark contrast, the predicted CYP1A2 correlation was not observed. The reason for this is unclear. Consistent with the recombinant P450 data, the correlation of OH-4 formation with CYP3A4 activity was highest. This analysis did not clearly identify the enzymes involved in the formation of the OH-3 and OH-5 metabolites (Supplementary Table S4).

In addition to the recombinant protein and probe substrate correlation analyses, we further characterized the P450s involved in osimertinib disposition using isoform-specific inhibitors in pooled HLM. In broad agreement with the above work, the generation of DM-1, DM-2, OH-1, and OH-2 were strongly inhibited by the CYP3A4 inhibitor ketoconazole (Supplementary Table S3). Further, in agreement with the correlation analysis, inhibition of CYP1A2 did not alter production of OH-2. Because of the reported low basal level of expression of CYP1A1 in human liver (28), we did not attempt to inhibit this enzyme in HLM.

To further characterize the involvement of CYP3A4 and CYP1A1 in osimertinib metabolism, we determined the apparent kinetic parameters for conversion to DM-1, DM-2, and OH-1 by recombinant forms of these enzymes in vitro. All data were in agreement with the above: (i) both CYP1A1 and CYP3A4 generated DM-1, with the former demonstrating a far lower $K_m$ and the latter demonstrating a higher $V_{max}$; (ii) CYP1A1 was essentially inactive in the formation of DM-2 with CYP3A4 exhibiting significant activity (iii) both enzymes generated OH-1, but CYP1A1 had both a lower $K_m$ and higher $V_{max}$ (Supplementary Table S4).

Osimertinib is an irreversible inhibitor of EGFR containing an acrylamide reactive center, which binds covalently to cysteine 797 in the kinase active site (5). Specificity for this target is enhanced through the formation of two hydrogen bonds between the pyrimidine core of osimertinib and methionine 793 of EGFR. This alpha/beta unsaturated ketone moiety is thiol-reactive (29), a common feature of many activators of the transcription factor nuclear erythroid 2 related factor 2 (NRF2). NRF2 provides a primary defense against reactive electrophiles and has been associated with drug resistance in cancer (30). We therefore tested the ability of osimertinib to activate NRF2-driven luciferase reporters in three stable cell lines; two driven off the endogenous heme oxygenase 1 (HO1) promoter, A549_HO1 and H1299_HO1, for which NRF2 is one of a number of regulators, and one driven off a synthetic promoter containing eight copies of the antioxidant response element (ARE), which is specific for NRF2, AREc32 (24).

In each cell line, treatment with 3 μmol/L osimertinib for 24 hours (a nontoxic dose, as determined by ATP cytotoxicity assay over 72 hours, data not shown) activated the reporter 2- to 3-fold (Supplementary Fig. S2).

Murine Cyp2d are responsible for the species difference in osimertinib metabolism

As described by Cross and colleagues, the half-life of oral clearance of osimertinib is approximately three hours in the mouse (5). However, in early clinical studies, the human value was found to be approximately 50 hours, "longer than would be
predicted from the preclinical data. The in vitro metabolic stability of osimertinib in HLM was much greater than in MLM (Fig. 3A). To determine the reason for this species difference in metabolism, we analyzed osimertinib stability in liver microsomes from Cyp2c, Cyp2d, and Cyp3a gene cluster knockout mice, and from a combined Cyp2c/2d/3a triple-cluster knockout line (19–22). Osimertinib stability was greatly increased in Cyp2d KO (Fig. 3A) and Cyp2c/2d/3a KO (not shown) microsomes but not in any of the other KO microsomes. The production of the major OH-2 metabolite was also concomitantly decreased (Fig. 3B). These data demonstrated that unlike in human samples, murine hepatic Cyp2d enzymes were responsible for osimertinib metabolism and that this defines a major species difference in the pathway of disposition. To establish whether this was also the case in vivo, and responsible for the short half-life of osimertinib in mice in vivo, we compared the PK profile of wild-type animals to those humanized for CYP2D6, which carry the human enzyme in place of the murine Cyp2d cluster. Consistent with the in vitro data, the humanized mouse model a profound change in osimertinib disposition was observed. The drug was cleared within 32 hours of administration to wild-type mice, yet remained detectable in serum from h2D6 animals 80 hours after administration (Fig. 3C). Exposure (AUC0–∞) and t1/2 were 5.3- and 2.8-fold higher, respectively, in the humanized animals, whereas Cl decreased 6.1-fold (Supplementary Table S5). Consistent with this, and with the in vitro data, the circulating OH-2 metabolite was also greatly decreased in the humanized line (Fig. 3D).

Osimertinib disposition following the induction of CYP3A4 in humanized mice in vivo

In humans, osimertinib is predominantly metabolized by CYP3A4 in the liver (25, 26, 31). We therefore investigated the role of this enzyme to osimertinib disposition in the humanized hPXR/hCAR/h3A4 mouse line in vivo. To induce hepatic CYP3A4 expression in this line, animals were pretreated with the PXR activator, Rif. To definitively attribute any effects observed to CYP3A4—that is, to rule out any effects of this inducing agent on, for example, drug transporters (23), we also carried out the analysis on a novel mouse line humanized for PXR and CAR, on a Cyp3a-knockout background. In the hPXR/hCAR/h3A4 line, pretreatment with Rif significantly decreased exposure to osimertinib, with a 2.4-fold decrease in AUIC0–∞ and a 2.1-fold decrease in Cmax (Fig. 4A and Supplementary Table S6). This decrease was due to the activity of CYP3A4, as no change was observed in the hPXR/hCAR/3aKO line with Rif pretreatment (Fig. 4B). Notably, both of the mouse models utilized here retain the Cyp2d gene cluster, hence the PK profile is more similar to wild-type mice than to h2D6.

In humans, CYP3A enzymes have been reported to be primarily responsible for both the generation and the further metabolism of the key active metabolites, DM-1 (AZ5104) and DM-2 (AZ7550; refs. 25, 26, 31). In the humanized model exposure to DM-1 decreased to a similar extent in both mouse lines after Rif pretreatment (Fig. 4C and D). This pattern was also apparent for DM-2, although was less well-defined due to the low signal intensity (Fig. 4E and F), suggesting that Rif induces a non-CYP3A4 route of disposition of these metabolites, possibly through the induction of drug transporters.

Osimertinib is a substrate and inducer of CYP1A1 in vivo

Preclinical data on the metabolism of osimertinib did not identify CYP1A1 as a catalyst of osimertinib metabolism (25, 26, 31, 32). As described above, recombinant CYP1A1 protein was highly active in generating DM-1, OH-1, and OH-5, and moderately active in generating DM-2, OH-2, and OH-3 (Fig. 2; Supplementary Table S4). These data are potentially important in relation to the outcome of osimertinib therapy as CYP1A1 is highly expressed in the lungs of smokers, and also in lung tumors (33). To explore the influence of CYP1A...
enzymes on osimertinib disposition in vivo, we carried out pharmacokinetic analysis in novel Cyp1a1/1a2 knockout and CYP1A1/1A2 humanized mouse lines. The basal expression of CYP1A1 in the h1A1/1A2 line is low (Kapelyukh and colleagues, manuscript in preparation), however it can be induced in a number of tissues including liver, lung, and small intestine by exposure of the mice to TCDD, an activator of the Ah receptor (Ahr). In h1A1/1A2 mice pretreated with TCDD, there was a 3.4-fold decrease in AUC0−t and a 3.3-fold decrease in Cmax of osimertinib (Fig. 5A and Supplementary Table S7). There was no change in exposure in the 1a1/1a2KO line. Correspondingly, TCDD-pretreatment greatly increased circulating levels of the OH-1 metabolite in humanized mice, but had no effect in knockouts (Fig. 5B). In this experiment, TCDD-mediated activation of Ahr occurred in several tissues—liver, small intestine, and lung (Kapelyukh and colleagues, manuscript in preparation)—hence the effects on osimertinib and metabolite disposition were likely to be driven by a combination of hepatic, intestinal, and pulmonary CYP1A1/1A2. To test whether induction of these enzymes in lung could alter osimertinib metabolism specifically in this tissue, we carried out in vitro studies using lung microsomes from h1A1/1A2, 1a1/1a2KO, and wild-type animals, both with and without TCDD-pretreatment (2 days prior to harvest). Induction of CYP1A1/1A2 in pulmonary microsomes from humanized animals increased OH-1 generation (Fig. 5C). Interestingly, there was no increase in OH-1 generation in samples for pretreated wild-type animals (Fig. 5C). There were, however, far greater increases in OH-3 (Fig. 5D) and OH-5 (Fig. 5E) production in lung samples from wild-type relative to h1A1/1A2 mice. As substrates of CYP1A1 are typically found also to be inducers of this enzyme, we administered osimertinib once daily for 4 days to h1A1/1A2 mice. Livers were harvested on day five and CYP1A1 protein levels assessed by Western blot analysis (Fig. 5F) and EROD activity (Fig. 5G). Increases in both indicated that this drug is indeed an inducer of CYP1A1 in vivo.
Discussion

Lung cancer is a global problem with high incidence and mortality. It is largely refractive to conventional chemotherapy and, despite large numbers of clinical trials and advances in stratification facilitated by increasing histopathologic subdivision, outcomes are poor. Recently, patients with activating mutations in EGFR have benefited from therapy with the first-generation EGFR inhibitors erlotinib and gefitinib (3, 4). However, resistance to these therapies rapidly emerges, with the T790M mutation in EGFR representing the predominant mechanism of innate/acquired resistance (8). Second-generation EGFR inhibitors designed to target this mutant protein exhibit significant side effects indicative of widespread inhibition of wild-type EGFR. As a consequence, the third generation of EGFR inhibitors has been designed with a higher level of specificity against the mutant forms of EGFR (5, 34). However, resistance to these drugs still emerges rapidly, the tumoral mechanism of which appears similar to those identified for the first-generation inhibitors (12–14). In this work we have identified and modeled, in humanized mice in vivo, the key metabolic factors which determine osimertinib disposition.

The half-life of oral clearance of osimertinib in wild-type mice is approximately 3 hours, yet, in the first human patients with confirmed radiographic response, half-life was observed to be approximately 50 hours, "longer than would be predicted from the preclinical data" (5). We have shown this species difference is due to the extremely high activity of enzymes in the murine Cyp2d gene family. Conversely, the human CYP2D6 homolog has minimal activity towards osimertinib. Knockout of the murine Cyp2d gene cluster (and humanization for CYP2D6) therefore renders both the in vitro metabolic stability and the in vivo PK profile of this drug much more representative of that observed in humans.

Unlike in mice, the primary pathway of osimertinib metabolism in patients is mediated by CYP3A enzymes (25, 26, 31). Indeed, prescribing guidelines advocate the avoidance of co-administration of strong CYP3A4 inducers. In clinical trials, the AUCss of osimertinib was reduced by 78% when combined with the CYP3A4 inducer rifampicin (35). We found that, in humanized PXR/CAR/CYP3A4 mice, rifampicin pretreatment also...
decreased osimertinib AUCₜ₀₋₉ by 58% and through the use of Cyp3a-knockout animals we confirmed that this was due to CYP3A4 induction. The osimertinib dose in our experiments gave a Cᵢₜ₀₋₉ of 123.6 ng/ml and an AUC₀₋₉ of 1144 hr·ng/ml. In humans, at the prescribed dose of 80 mg once daily, Cᵢₜ₀₋₉ was 311.7 ng/ml (623.8 nmol/L [range: 167–2100, CV: 53.84%]) and AUC₀₋₉ was 5960 hr·ng/ml (11930 nmol·hr/L [range: 3650–38900, CV: 51.77%]) (15). Hence, although our studies gave levels of exposure in the lower range of clinically-observed values, the quantitative similarity with the human data demonstrate that CYP3A4-humanized animals provide a powerful model for the prediction of CYP3A4-mediated effects on human PK and drug efficacy.

Metabolism by CYP3A4 generates two pharmacologically active metabolites, AZ5104 and AZ7550 (here referred to as DM-1 and DM-2, respectively), which have selectivity profiles similar to parent compound, although the former is less specific for T790M (25, 26, 31). These metabolites are, themselves, further metabolized by CYP3A4/5. In our study, pretreatment with rifampicin decreased exposure to AZ5104 and AZ7550 in both mouse lines. These data suggest that Rif is acting through PXR, to induce a novel CYP3A4-independent pathway involved in disposition of these pharmacologically active metabolites, possibly through the induction of the drug transporters.

Although CYP3A4 is reported to be the major enzyme responsible for osimertinib metabolism, it is important to note that intra- and inter-individual variability in the hepatic levels of this enzyme can be as high as 60-fold (27). In this paper, we report the identification of another novel pathway of osimertinib metabolism involving CYP1A1 and that suggest that this enzyme may also play an important role in both osimertinib disposition and in its efficacy. CYP1A1 generated both the demethylated and hydroxylated metabolites of osimertinib and, indeed, had the highest activity in the generation of OH-1, OH-3, and OH-5 metabolites. CYP1A1 is a highly inducible enzyme in almost all tissues as a consequence of activation of the AhR, most notably by the polycyclic aromatic hydrocarbons (PAH) found in cigarette smoke (28, 33). Further, it is often expressed at significant levels in tumors (36). CYP1A2 is considered more liver-specific, but its promoter contains an element responsive to PAHs (37) and smoking alters the disposition of a wide variety of drugs and, in some cases, this interaction requires dose adjustment or a change in the drug prescribed (39).

CYP1A1-mediated metabolism is of particular relevance to the treatment of lung cancer, as this enzyme may metabolize drugs within lung tumors or may alter drug exposure due to metabolism in the adjacent lung tissue. This possibility is exemplified by our finding that in vitro incubation of osimertinib with lung microsomes following the activation of AhR and the induction of CYP1A1, led to greatly increased production of the OH-1 metabolite, with some increase in the OH-3 or OH-5 osimertinib metabolites. Intratumoral osimertinib metabolism could affect both drug and metabolite levels and therefore contribute to individual patient responses or indeed drug resistance.

The induction of CYP1A1 in the lung has also been shown to alter drug exposure (40). In the case of the CYP1A2 substrate, erlotinib, a dose adjustment is recommended in smokers to counteract the increased clearance mediated by CYP1A1/1A2 (41). Recently, phase I clinical trial of a combination of erlotinib with drotinib had to be halted due to a high incidence of grade 3 toxicity and a profound drug–drug interaction (DDI), stemming from drotinib acting as a CYP1A1/1A2 inducer (42). Using a novel CYP1A1/1A2 humanized mouse line, we found that induction of these enzymes following systemic activation of AhR greatly decreased exposure to osimertinib. Brown and colleagues, in a population PK analysis of 778 patients, found that smoking status (current smokers = 3%, former smokers = 30%, never smokers = 67%) did not have a significant effect on osimertinib PK (dose-normalized AUCᵢₜ₀₋₉ concluding that CYP1A1 induction does not have a major effect on metabolism (16). However, the very small number of current smokers in this study, and the fact that these data were not analyzed in relation to when smoking cessation occurred, makes interpretation of the potential effects on osimertinib pharmacokinetics unclear. This is an important issue in relation to our work, because when patients stop smoking is a key factor in determining CYP1A1 levels. In normal lung tissue of patients who stop smoking, CYP1A1 levels begin to decrease after two weeks, becoming undetectable after 6 weeks (33). Initially, the labeling of osimertinib recommended the avoidance of co-administration of substrates of CYP1A due to potential induction of CYP1A2 (25, 32). This recommendation was removed from the label in August 2016, without explanation (35). Our data suggest that the interaction of osimertinib with CYP1A1/1A2 could have significant effects on its therapeutic efficacy. We have examined the AURA clinical trial data, including the recently published FLAURA study, to evaluate evidence that smoking is a factor in the outcome of therapy. This has not been possible because detailed information on smoking history—in particular, time from cessation in relation to the initiation of treatment—was not reported. This information should now be gathered as part of the approved clinical use of osimertinib.

During clinical trial of osimertinib, the 80 mg per day dose level was selected for evaluation in phase II as, above this level, incidence and severity of adverse events increased (15). There is no evidence of an exposure/response relationship for efficacy across the 20 to 240 mg dose range. There is however a strong relationship between dose and toxicity, with higher exposure resulting in increased likelihood of rash, diarrhea—adverse events indicative of the inhibition of wild-type EGFR—or elongation of the QTc interval (15, 16). Therefore, it would appear that a dose reduction is feasible, and might reduce the high incidence of grade 1 and 2 adverse events (85%; ref. 11) without loss of efficacy. At steady-state, inter-individual variability in osimertinib PK is high: the coefficient of variation for Cᵢₜ₀₋₉ is 54%, whereas for AUC₀₋₉ is 52% (43). Individuals at either end of the spectrum may be greatly under- or overexposed to drug. Much of this variability in exposure may be driven by variability in CYP3A4 and CYP1A1 expression levels. Notably, Brown and colleagues identified ethnicity as a covariate for AUC of the CYP3A-generated metabolite, AZ5104 (DM-1), with lower levels observed in non-Caucasian patients, including Japanese (16). One possible explanation for this may be the lower level of CYP3A activity in Japanese men than in European American men (44). Should this be found also to be the case in the other populations examined (Chinese, non-Chinese/non-Japanese Asian, and non-Asian non-Caucasian). It would be interesting to determine whether or to what extent P450 levels and exposure to osimertinib and its active metabolites correlate with degree of clinical response, severity of side-effects, the emergence of resistance and disease progression, especially in light of our observation that, at a concentration which reflects that
achieved clinically, this drug activates adaptive cellular stress responses which might alter its intracellular levels.

There are several indications that osimertinib may be suitable for first-line therapy of lung tumors; a low incidence of grade 3 or above adverse events (15), probable high levels of brain penetration with resultant enhanced efficacy over existing EGFR inhibitors for the treatment of metastases and leptomeningeal carcinomatosis (5, 25, 45, 46), and, although the frequency is contentious, the preexistence of T790M-positive clones in TKI-naive EGFRm+ tumors (47). Moreover, there are many combination strategies involving osimertinib either proposed or in trial. As detailed above, RAS mutations are thought to confer resistance to osimertinib, which suggests a potential combination with MEK inhibitors (12). Other identified resistance mechanisms involve EGFR bypass through HER2/ERBB2 or MET amplification/activation, similar to the T790M-unrelated mechanisms which occur in response to first-line germline EGFR-mutant tumors (12, 13), and treatment with MET or EPHA2 inhibitors may be effective in these cases (48, 49). Furthermore, although overexpression of ABCB1 in cell lines did not confer resistance to osimertinib, this compound inhibits ABCB1 activity and can enhance the efficacy of concomitantly administered cytotoxic agents (50). We posit that mice humanized for pathways of drug disposition can be used to model and predict drug–drug interactions prior to clinical trial. Such application would aid in the identification and prioritization of the combination doses and schedules most likely to succeed in the clinic.

In conclusion, the data we have presented here will aid in the more effective deployment of osimertinib through pharmacologically-guided dose-adjustment. Moreover, with the ever-increasing molecular stratification of lung cancer suggesting further options for targeted intervention, and the expanding arsenal of targeted agents which might be combined with osimertinib, knowledge of its PK and DDI profiles will be essential for the pharmaceutically-guided design of effective and tolerable combinations.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: A.K. MacLeod, D. Lin, C.J. Henderson, C.R. Wolf
Development of methodology: A.K. MacLeod, D. Lin, L.A. McLaughlin
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.K. MacLeod, D. Lin, J.T–J. Huang, L.A. McLaughlin, C.J. Henderson
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.K. MacLeod, D. Lin, J.T–J. Huang, C.R. Wolf
Writing, review, and/or revision of the manuscript: A.K. MacLeod, D. Lin, C.J. Henderson, C.R. Wolf
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D. Lin
Study supervision: J.T–J. Huang, C.J. Henderson

Acknowledgments
We thank Dr. Yury Kapelyukh for conducting the EROD assay and Dr. Shaohong Ding for carrying out the cell line work. We would also like to acknowledge Taconic Biosciences for the supply of the mouse lines used in this study. This work was supported by Cancer Research UK programme grant C4639/1/A10822.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received November 29, 2017; revised January 17, 2018; accepted January 29, 2018; published first February 6, 2018.

References


25. FDA, CPBR. http://www.accessdata.fda.gov/drugsatfda_docs/nda/.../208065Orig1s000ClinPharmR.pdf.
32. FDA. PR. http://www.accessdata.fda.gov/drugsatfda_docs/nda/2015/ 208065Orig1s000PharmR.pdf.
Identification of Novel Pathways of Osimertinib Disposition and Potential Implications for the Outcome of Lung Cancer Therapy

A. Kenneth MacLeod, De Lin, Jeffrey T.-J. Huang, et al.

Clin Cancer Res 2018;24:2138-2147. Published OnlineFirst February 6, 2018.

Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-17-3555

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2018/02/06/1078-0432.CCR-17-3555.DC1

Cited articles
This article cites 44 articles, 18 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/24/9/2138.full#ref-list-1

Citing articles
This article has been cited by 4 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/24/9/2138.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://clincancerres.aacrjournals.org/content/24/9/2138.
Click on "Request Permissions" which will take you to the Copyright Clearance Center’s (CCC) Rightslink site.