

Exploring the mechanism of CYP3A4 inactivation by lapatinib through *in vitro* metabolite characterization

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Overview

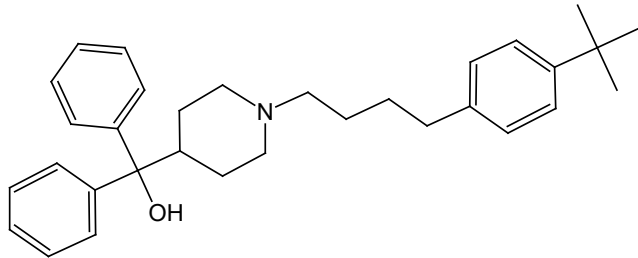
- Cytochrome P450 (CYP) inhibition in drug development
- Metabolite profiling in drug development
 - Questions addressed
 - Accurate mass spectrometry
- Mechanistic studies in enzymatic inactivation caused by lapatinib

Cytochrome P450 inhibition

- Cytochrome P450 (CYP) enzymes mediate oxidation of over half of hepatically-cleared drugs
- Some drugs interact with the proteins to cause CYP inactivation
 - Direct inhibition (drug)
 - Metabolism-dependent inhibition (metabolite)
- This can lead to clinically-relevant drug-drug interactions

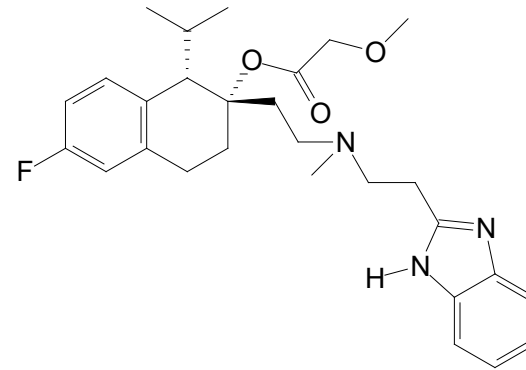
Cytochrome P450 inhibition

- CYP inhibition has potential to result in
 - Black box label warnings
 - Withdrawal from market



Terfenadine: withdrawn 1997
(victim drug)

Co-administration with CYP3A4 inhibitors (e.g., ketoconazole) reduces clearance of the drug and results in cardiotoxicity caused by terfenadine accumulation

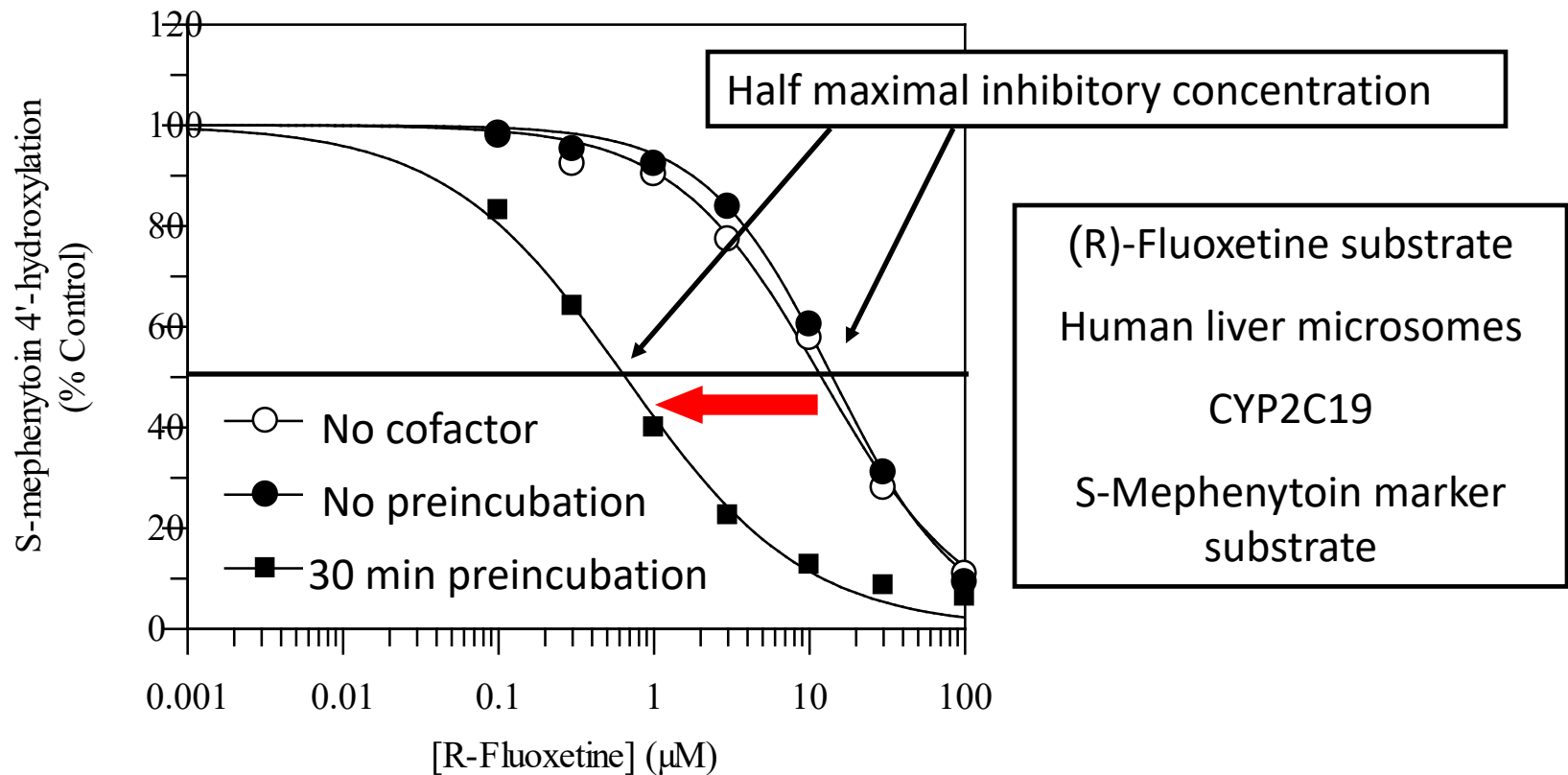


Mibefradil: withdrawn 1998
(perpetrator drug)

Mibefradil inhibits CYP3A4 and CYP2D6 and can cause elevated levels of coadministered drugs cleared by these enzymes. Life-threatening interactions can occur with β -blockers and other antihypertensives

Establishing metabolism-dependent inhibition

- Measure effect of pre-incubation with the test article on the *in vitro* half maximal inhibitory concentration (IC_{50}) of known specific substrates for each relevant CYP enzyme

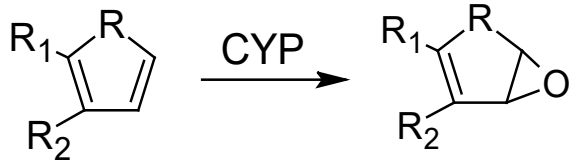


Metabolism-dependent cytochrome P450 inhibition (MDI)

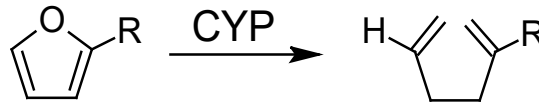
- Three main categories
- Reversible inhibition
 - Metabolite is a potent direct CYP inhibitor
- Irreversible inhibition
 - Metabolite binds covalently to the heme or apoprotein
- Quasi-irreversible inhibition
 - Inhibitory metabolite forms a stable metabolic intermediate (MI) complex with the ferrous heme iron

Functional groups associated with metabolism-dependent inhibition

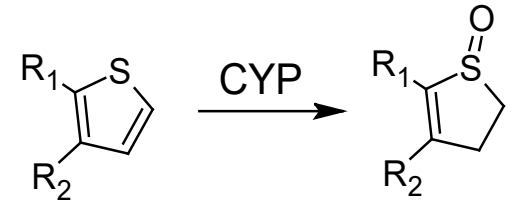
Several functional groups are associated with metabolism-dependent inhibition



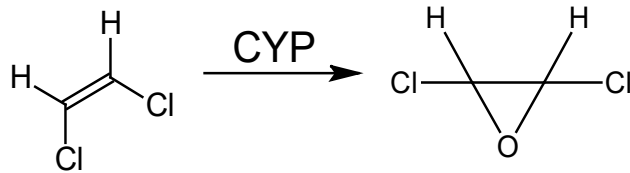
Furans/thiophenes/multiple bonds



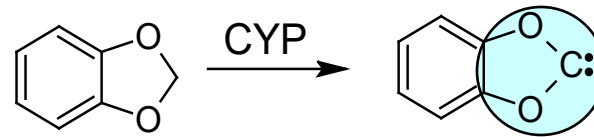
Furans/methylfurans



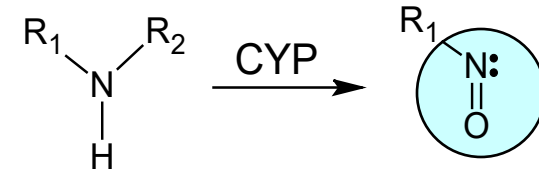
Thiophenes



Di/trichloroethylenes



Benzodioxoles

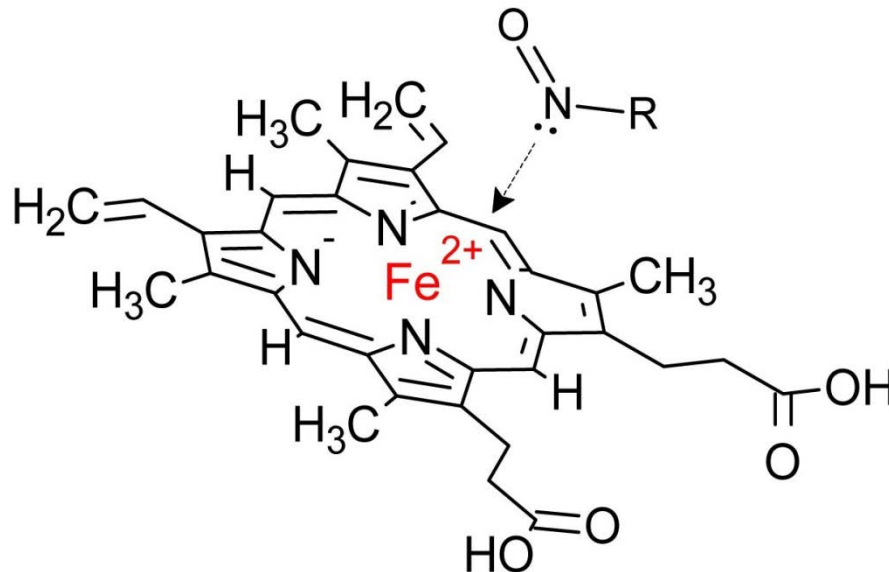


Alkylamines

But only these two are associated with quasi-irreversible inhibition

Quasi-irreversible inhibition

- Based on a noncovalent interaction with the ferrous heme iron (Fe^{2+})
- MI complex stabilized by a co-ordinate bond formed between the iron and an electron pair donor

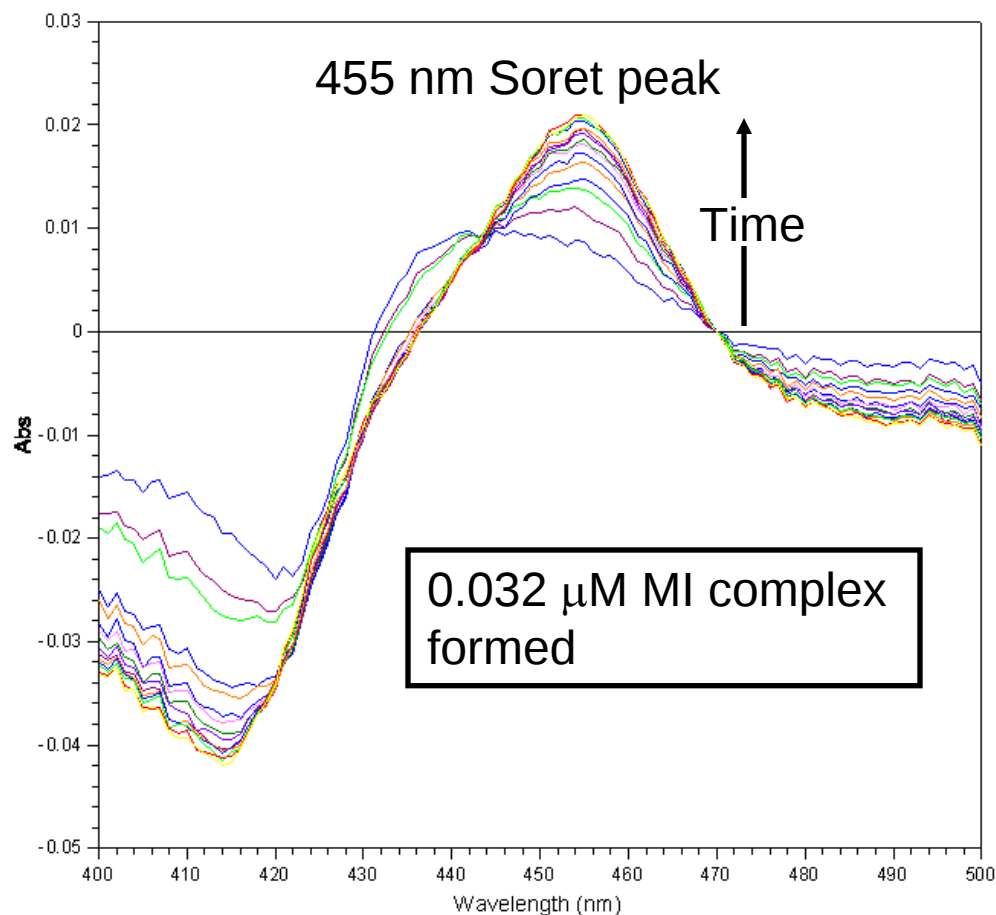


- Can be detected with optical spectrophotometry ($\lambda_{\text{max}} \sim 455 \text{ nm}$)
- Complex can be reversed by oxidizing the iron to the ferric state (Fe^{3+}) with a chemical oxidizer (e.g., ferricyanide)

MI Complex detection by optical spectrophotometry

Troleandomycin MI complex formation

- Human liver microsomes
- 100 μM troleandomycin
- 0.1 mg/mL protein
- pH 7.4, 37°C
- 8 min incubation

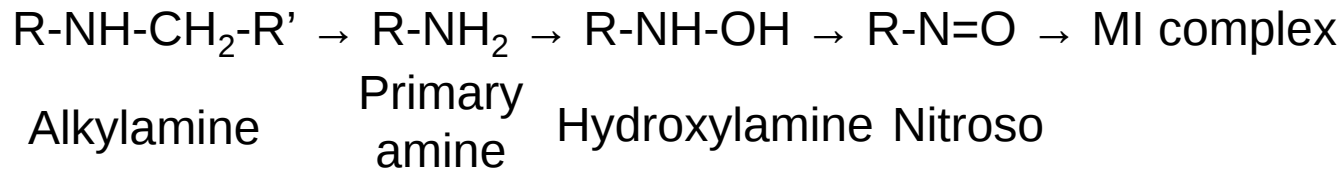


Quasi-irreversible inhibition associated with alkylamines

- Secondary/tertiary alkylamines can cause quasi-irreversible inhibition

| | | | |
|---------------|---------------------------|------------------------------|---------------------|
| NH_3 | $\text{CH}_3\text{-NH}_2$ | $\text{CH}_3\text{-NH-CH}_3$ | $\text{N-(CH}_3)_3$ |
| Ammonia | Primary amine | Secondary amine | Tertiary amine |

- One proposed mechanism involves

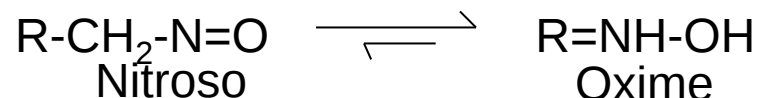


- Alkylamine drugs including macrolide antibiotics, antidepressants (SSRIs and tricyclics), antihistamines and calcium-channel blockers known for MI complex formation with several CYP enzymes

Evidence of involvement of a nitroso metabolite

- First step in MI complex formation by tertiary alkylamine is initiated by N-dealkylation
 - SKF 525-A (Proadifen) Schenkman et al., (1972) *Biochem Pharmacol* **21**: 2373-2383.c
 - Macrolide antibiotics
Danan et al., (1981) *J Pharmacol Exp Ther* **218**: 509-514. Pessayre et al., (1981) *Biochem Pharmacol* **30**: 553-558.
Pessayre et al., (1982) *Biochem Pharmacol* **31**: 1699-1704.
- Electron pair donor proposed as nitroso metabolite
 - Heme model/model protein-based studies
Mansuy et al., (1976) *Biochem Pharmacol* **25**: 609-612. Mansuy et al., (1977) *Eur J Biochem* **76**: 617-623.
Mansuy et al., (1977) *J Am Chem Soc* **99**: 6441-6443. Mansuy et al., (1981) *Biochem Toxicol* **3**: 283-320.
 - Metabolite involved in MI complex derived from both hydroxylamine oxidation and nitro reduction Mansuy et al., (1978) *Eur J Biochem* **86**: 573-579.
- Computation showed MI complex formed with nitroso exothermic (-60 kcal mol⁻¹)
Taxak et al., (2012) *J Comput Chem* **33**: 1740-1747.
- First report of a nitroso/oxime metabolite formed from a drug associated with MI complexation was recently established

Takakusa et al., (2011) *Drug Metab Dispos* **39**: 1022-1030.



In vitro metabolite profiling in discovery/development

- *In vitro* species metabolite profiling and characterization can
 - Aid lead compound structure optimization (soft spots)
 - Determine potentially active or toxic metabolites
 - Aid design/data interpretation for animal toxicology studies
 - Provide information on the drug metabolizing enzymes relevant to the drug

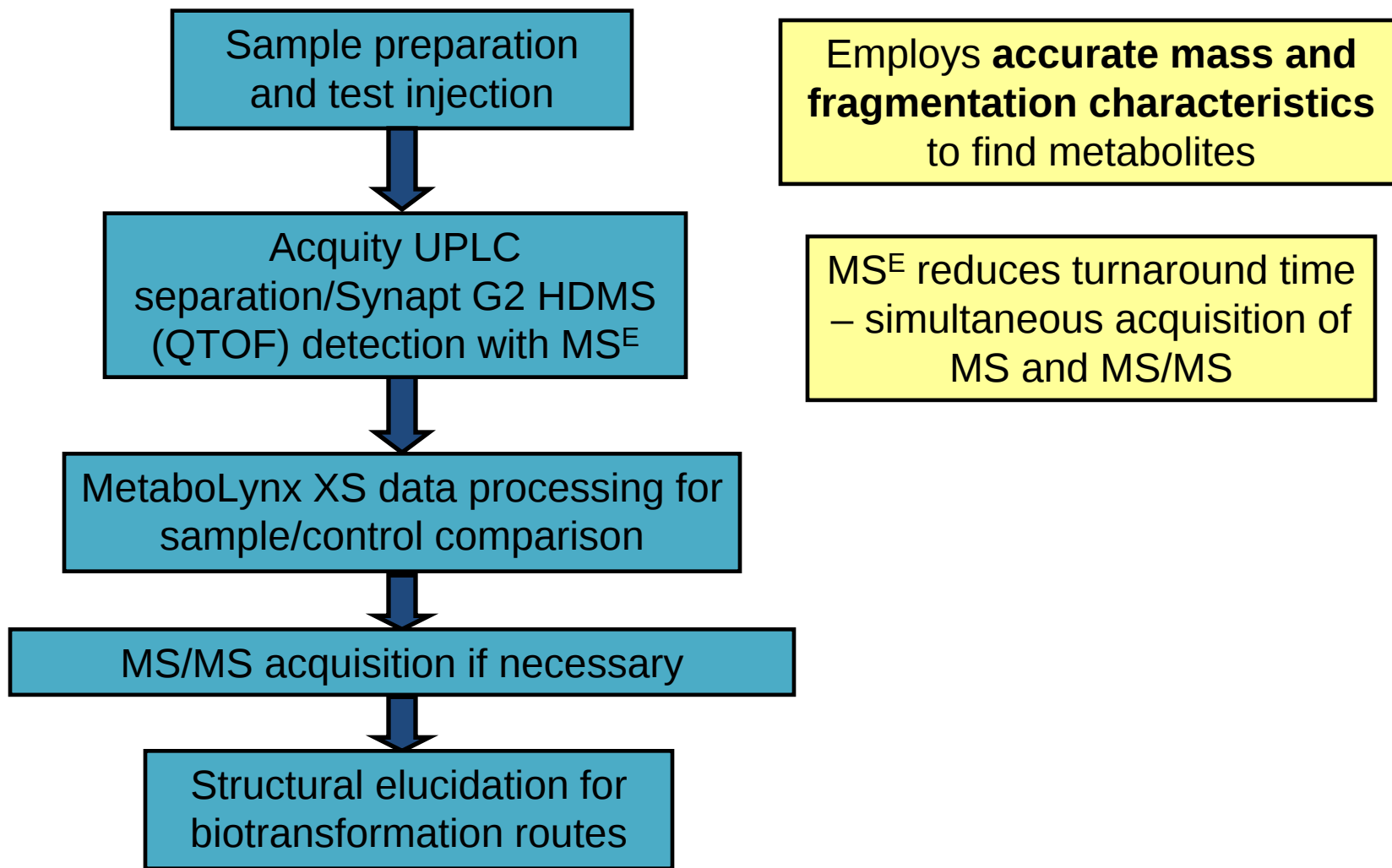
In vitro metabolite profiling experiments

- Incubate parent drug with appropriate test system
- Separate metabolites and matrix components by extraction and/or chromatography
- Detect metabolites by various techniques
 - Optical spectrophotometric detection (UV/vis, fluorescence)
 - Radiometric detection (^{14}C , ^3H , ^{35}S)
 - Mass spectrometric detection
- Perform structural elucidation of detected components with mass spectral data

Accurate mass spectrometry for metabolite profiling

- Accurate (or high-resolution) mass spectrometry is the approach of choice for metabolite profiling
 - Waters Synapt G2 high-definition (HD) MS system (quadrupole time-of-flight)
- Accurate mass measurements enhance structural characterization capabilities
- The associated workflow is efficacious for the *a priori*, rapid characterization of unknowns
 - No prior assumptions about metabolite structure

Accurate mass spectrometry approach



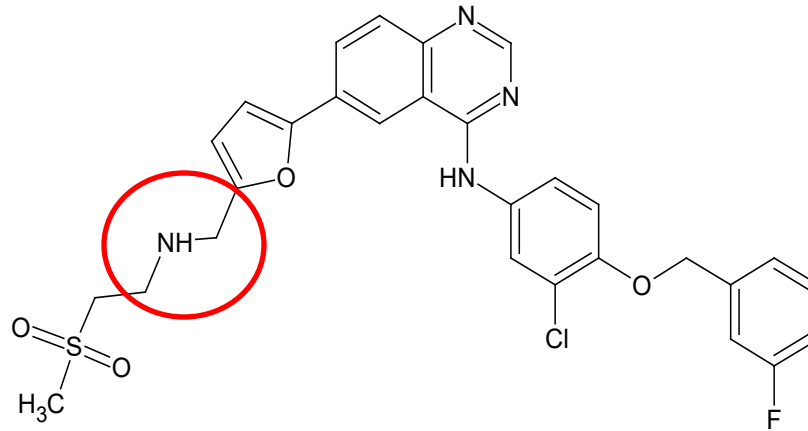
Accurate mass spectrometry benefit

- Accurate mass data aids biotransformation assignment

| Nominal mass shift (amu) | Biotransformation(s) | Elemental composition change | Accurate mass shift (amu) |
|--------------------------|--|------------------------------|---------------------------|
| +14 | Oxidation + dehydrogenation | +O -2H | +13.9792 |
| | Methylation | +CH ₂ | +14.0157 |
| +16 | Oxidation | +O | +15.9949 |
| | Heteroatom dealkylation within a heterocycle | | |
| +18 | Oxidation + hydrogenation | +O +2H | +18.0106 |
| | Hydrolysis | +H ₂ O | |
| +28 | 2x (Oxidation + dehydrogenation) | +2O -4H | +27.9584 |
| | Oxidation + dehydrogenation + methylation | +O -2H +CH ₂ | +27.9949 |
| | Di-methylation or ethylation | +2(CH ₂) | +28.0313 |
| +32 | Di-oxidation | +2O | +31.9898 |
| | Oxidation + hydrogenation + methylation | +O +2H +CH ₂ | +32.0263 |

The alkylamine drug lapatinib

- Lapatinib is a tyrosine kinase inhibitor



- Metabolized by CYP3A4
- Causes quasi-irreversible inhibition of CYP3A4
- Many alkylamine drugs are associated with quasi-irreversible inhibition of CYP enzymes
- Lapatinib also forms stable N-oxygenation metabolites

Hypothesis

In vitro incubation with ultracentrifugation, chemical oxidation by ferricyanide, and liquid chromatography/tandem mass spectrometry-based metabolite profiling could identify the complexed lapatinib metabolite(s) causing the CYP3A4 inhibition

Metabolism-dependent inhibition of CYP3A4 by lapatinib: Evidence for formation of a metabolic intermediate complex with a nitroso/oxime metabolite formed via a nitron intermediate

Joanna E. Barbara, Faraz Kazmi, Andrew Parkinson, David B. Buckley

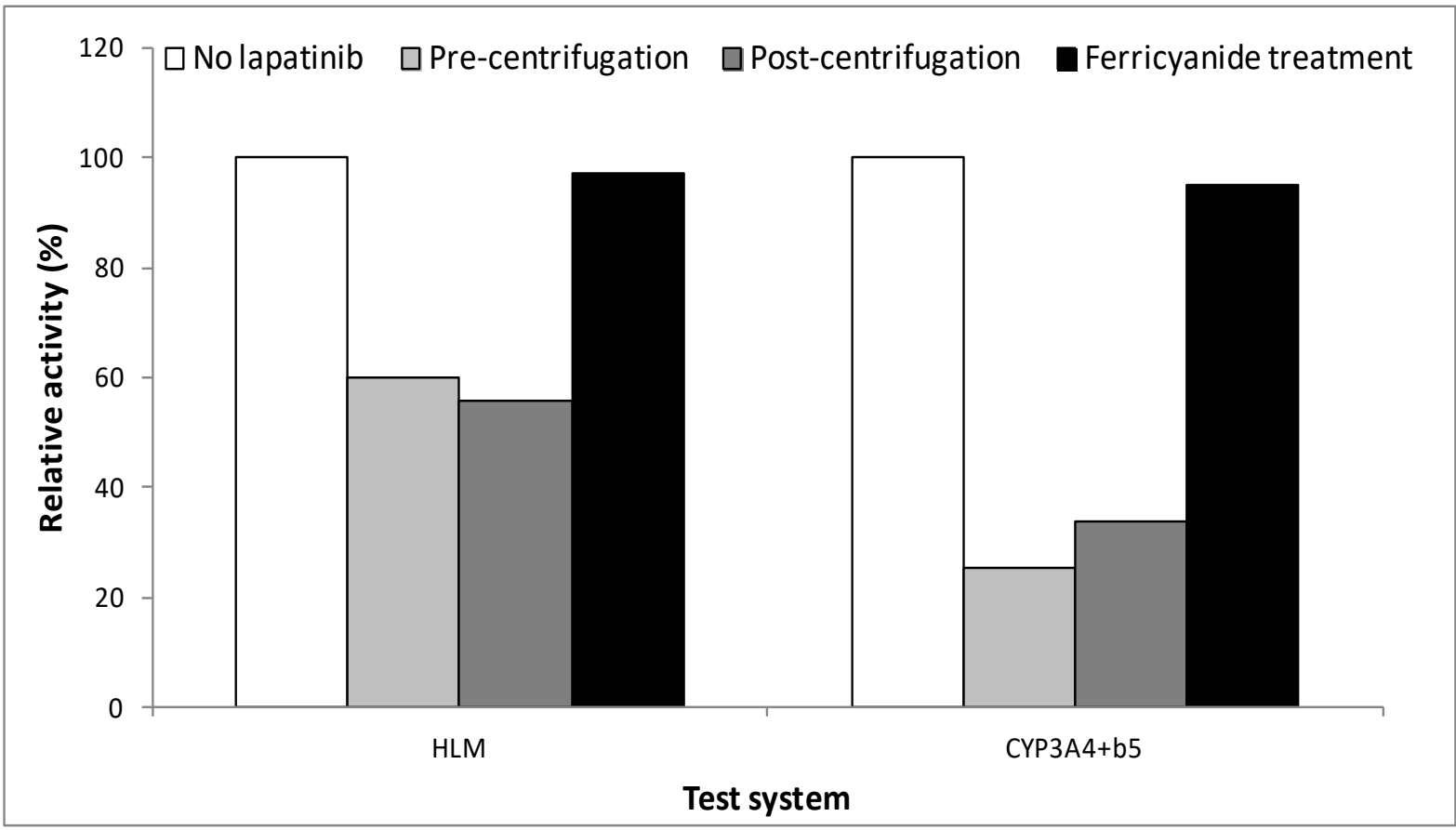
Drug Metabolism and Disposition

Epub ahead of print 12 February, 2013

doi: 10.1124/dmd.113.051151

Reversibility assessment of CYP3A4 inhibition by lapatinib

- Human liver microsomes/rCYP3A4; Midazolam

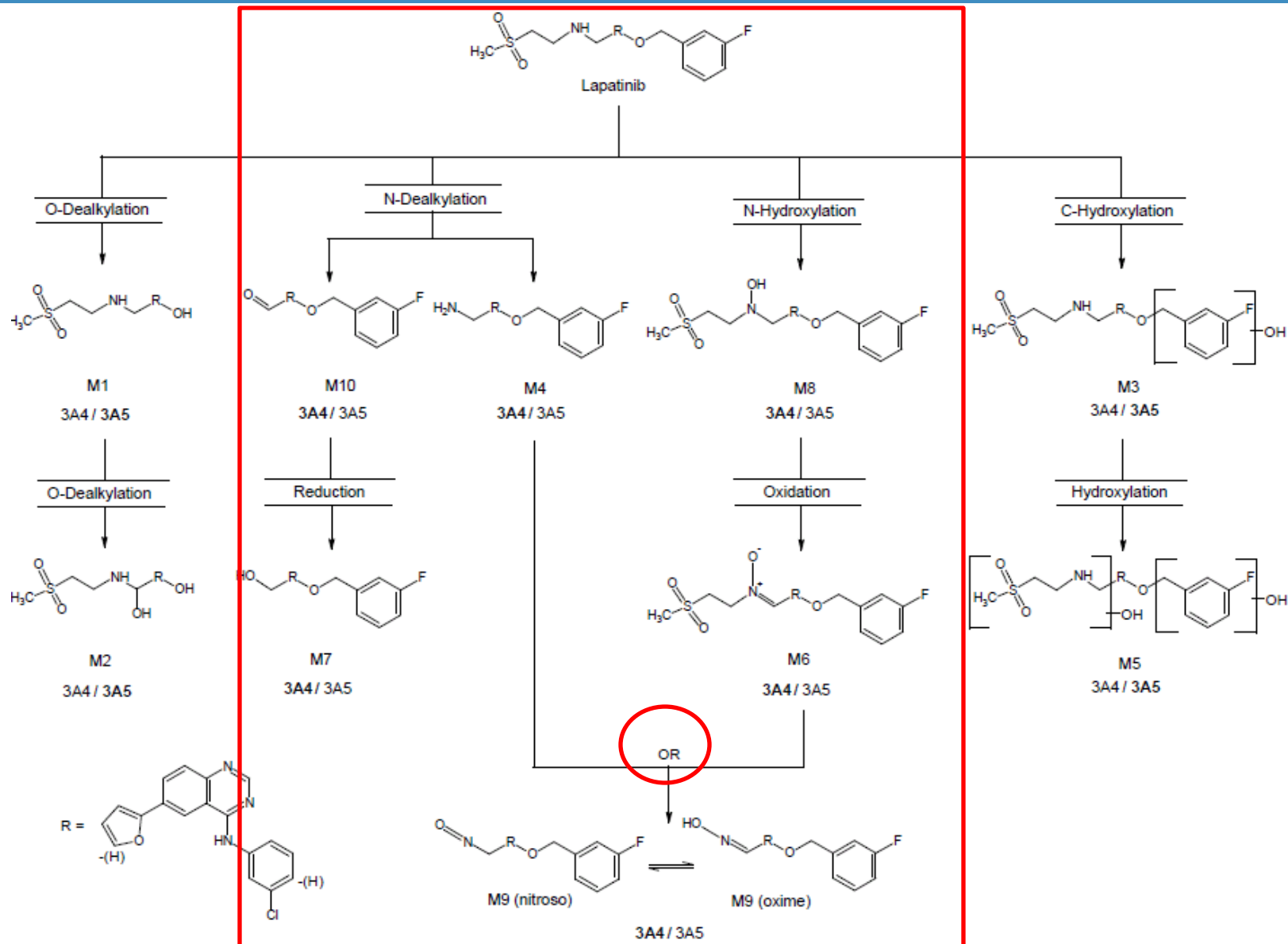


Lapatinib metabolites in HLM, CYP3A4 and CYP3A5

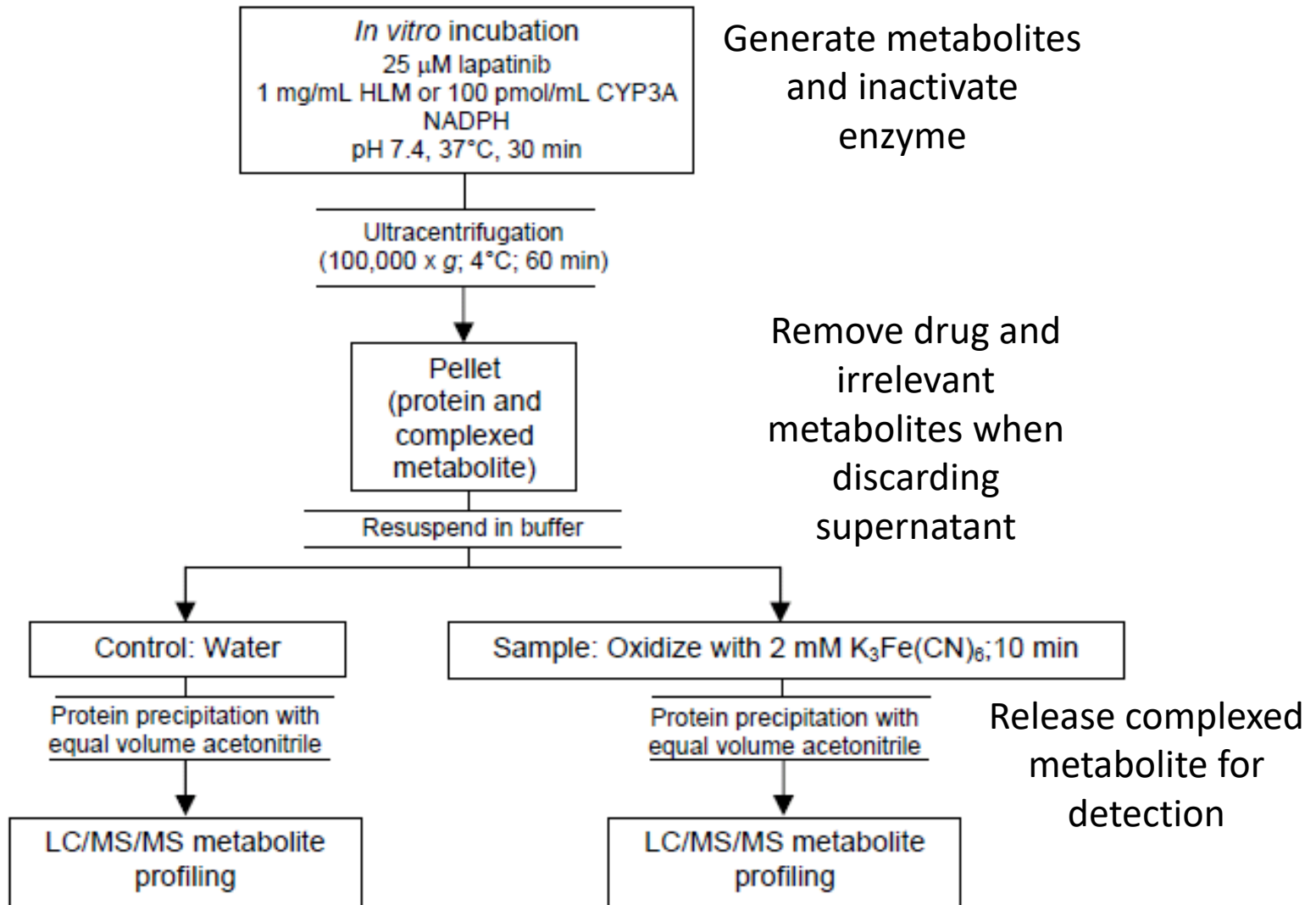
- No quasi-irreversible inhibition of CYP3A5 (negative control)

| Name | $t_{r,rel}$ | $[M+H]^+$ exp | Elemental composition | Mass error (ppm) | Proposed biotransformation |
|-----------|-------------|------------------|--------------------------|------------------------|---|
| M1 | 0.50 | 473.1052 | $C_{22}H_{21}ClN_4O_4S$ | 0.4 | O-Dealkylation |
| M2 | 0.78 | 489.0995 | $C_{22}H_{21}ClN_4O_5S$ | -0.8 | O-Dealkylation + oxygenation |
| M3 | 0.95 | 597.1367 | $C_{29}H_{26}ClFN_4O_5S$ | -1.3 | Oxygenation |
| M4 | 0.97 | 475.1336 | $C_{26}H_{20}ClFN_4O_2$ | -0.2 | N-Dealkylation to amine |
| Lapatinib | 1.00 | 581.1427 | $C_{29}H_{26}ClFN_4O_4S$ | 0.2 | N.A. (Parent) |
| M5 | 1.10 | 613.1331 | $C_{29}H_{26}ClFN_4O_6S$ | 1.1 | Dioxygenation |
| M6 | 1.11 | 595.1220 | $C_{29}H_{24}ClFN_4O_5S$ | 0.3 | Oxygenation + further oxidation |
| M7 | 1.13 | 476.1176 | $C_{26}H_{19}ClFN_3O_3$ | -0.2 | N-Dealkylation to aldehyde + reduction |
| M8 | 1.14 | 597.1368 | $C_{29}H_{26}ClFN_4O_5S$ | -1.2 | Oxygenation |
| M9 | 1.18 | 489.1125 | $C_{26}H_{18}ClFN_4O_3$ | -1.0 | N-Dealkylation to amine + oxygenation + oxidation |
| M10 | 1.21 | 474.1016 | $C_{26}H_{17}ClFN_3O_3$ | -1.1 | N-Dealkylation to aldehyde |

Lapatinib biotransformation in HLM, rCYP3A4 and rCYP3A5

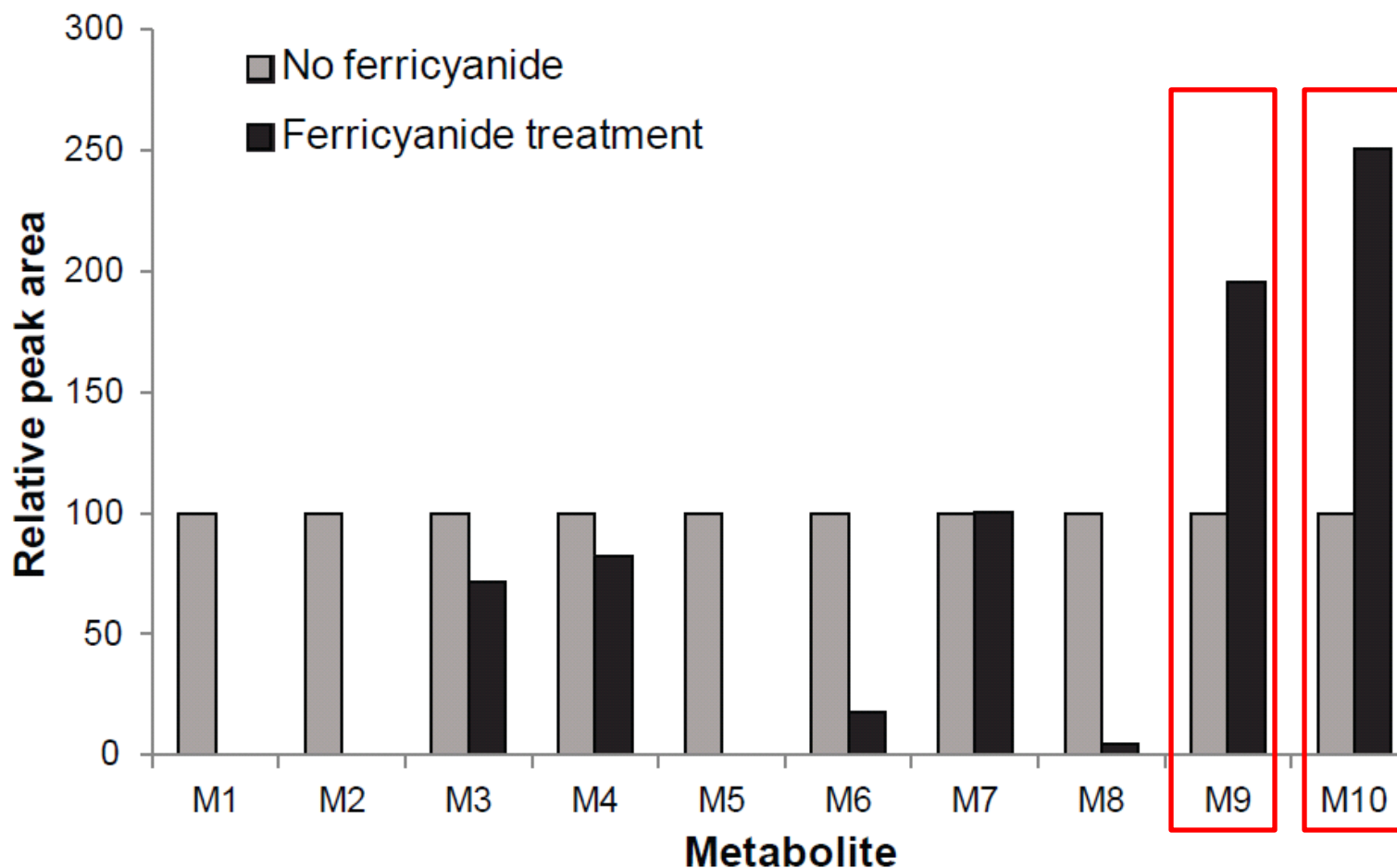


Experimental method



Ferricyanide effect on metabolite formation in HLM

- M9 and M10 increased with ferricyanide oxidation



CYP3A4 and CYP3A5 lapatinib inhibition differences

- Lapatinib is quasi-irreversible inhibitor of CYP3A4 (primarily N-dealkylation metabolism)

Takakusa et al., (2011) *Drug Metab Dispos* **39**: 1022-1030.

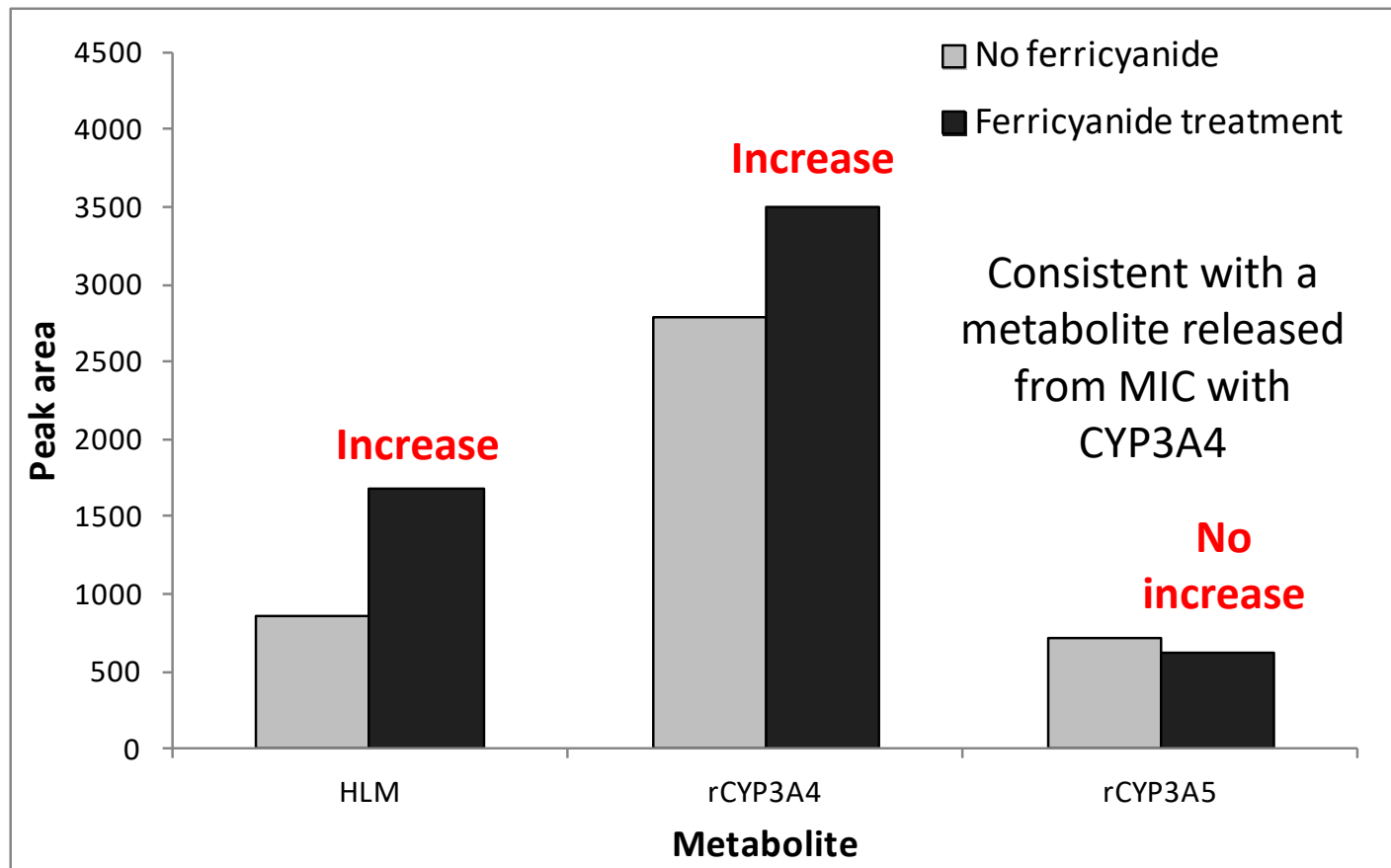
- Lapatinib is irreversible inhibitor of CYP3A5 (primarily O-dealkylation metabolism)

Chan et al., (2012) *Drug Metab Dispos* **40**: 1414-1422.

- We can use that difference to link metabolites to quasi-irreversible inhibition

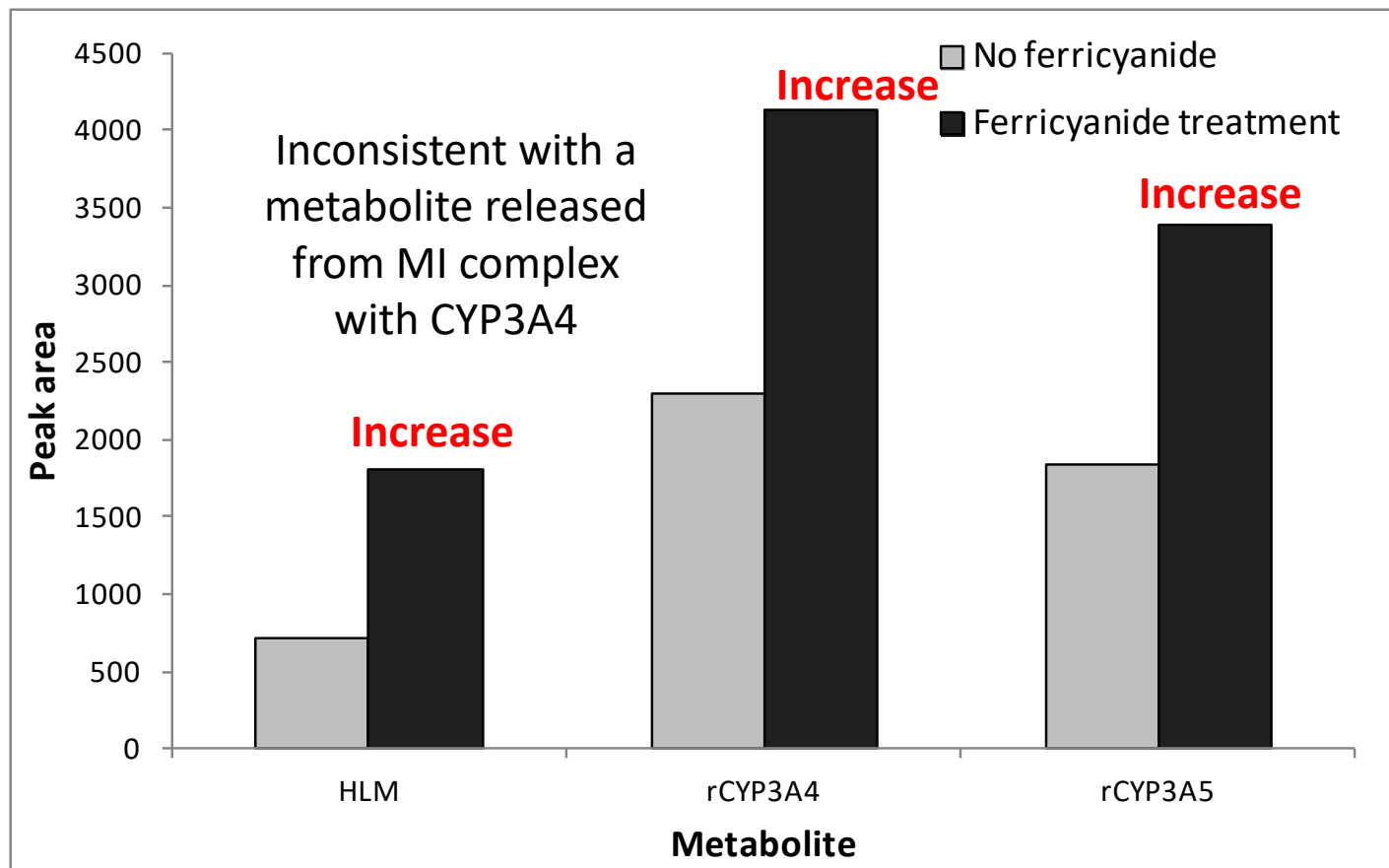
Recombinant CYP3A4 *versus* CYP3A5: M9

- Effects of oxidation differ between the test systems



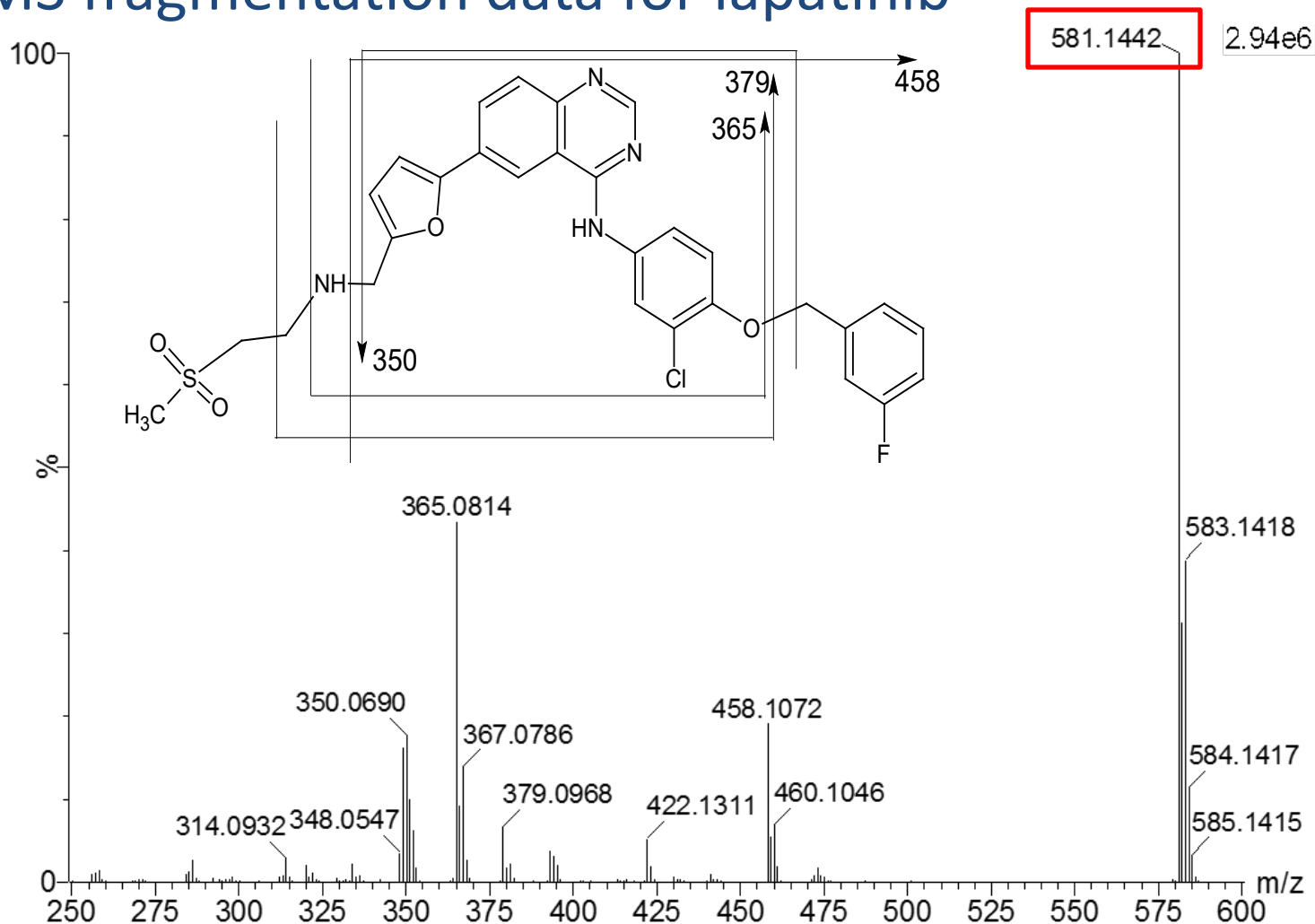
Recombinant CYP3A4 *versus* CYP3A5: M10

- Effects of oxidation show no test system differences

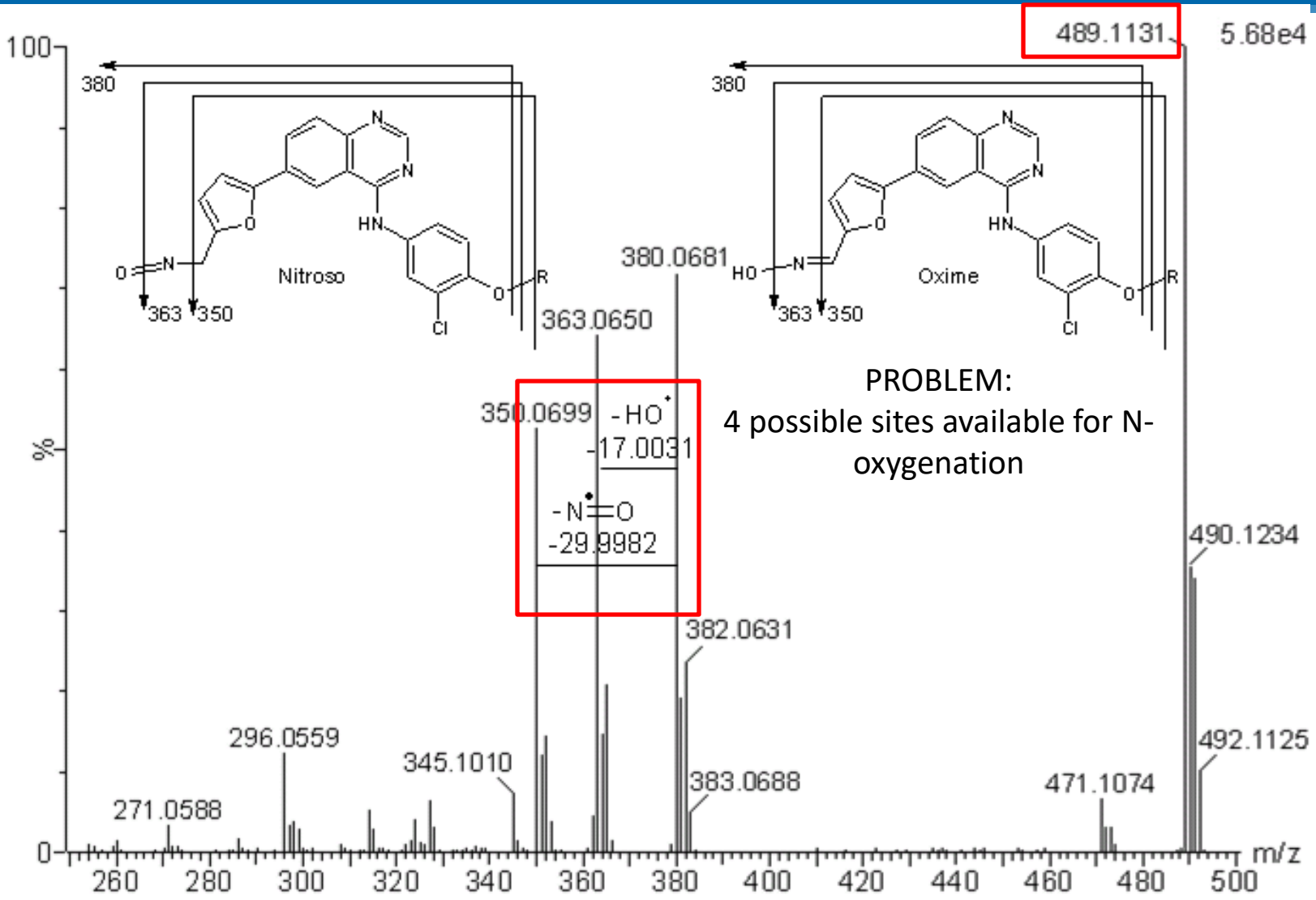


Identifying M9: Structural elucidation

- MSMS fragmentation data for lapatinib

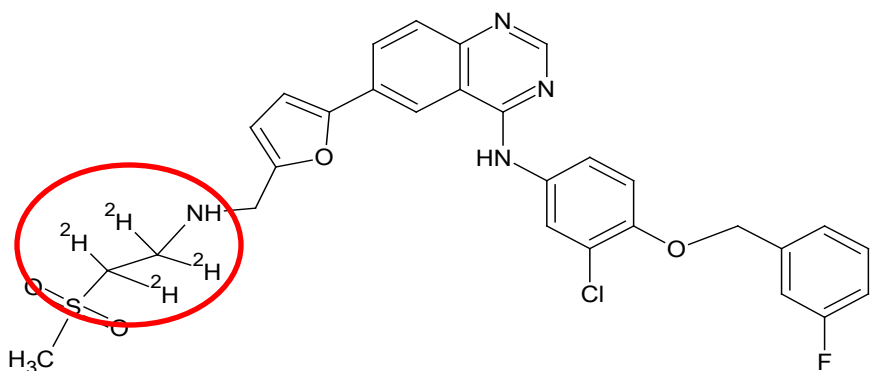


M9 MSMS fragmentation data with proposed assignments



Stable-isotope labeling experiments

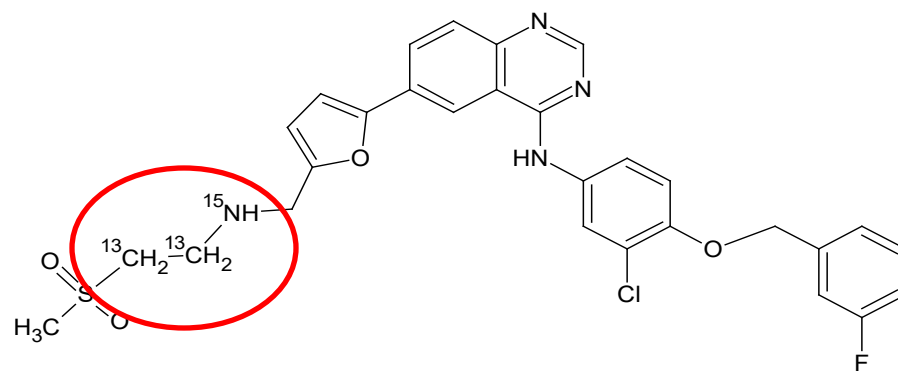
- No metabolite standards available
- $^2\text{H}_4$ -lapatinib and $^{13}\text{C}_2, ^{15}\text{N}$ -lapatinib were available



$^2\text{H}_4$ -Lapatinib

Molecular Formula = $\text{C}_{29}^2\text{H}_4\text{H}_{22}\text{ClFN}_4\text{O}_4\text{S}$
Monoisotopic Mass = 584.159838 Da

+4 from unlabeled lapatinib



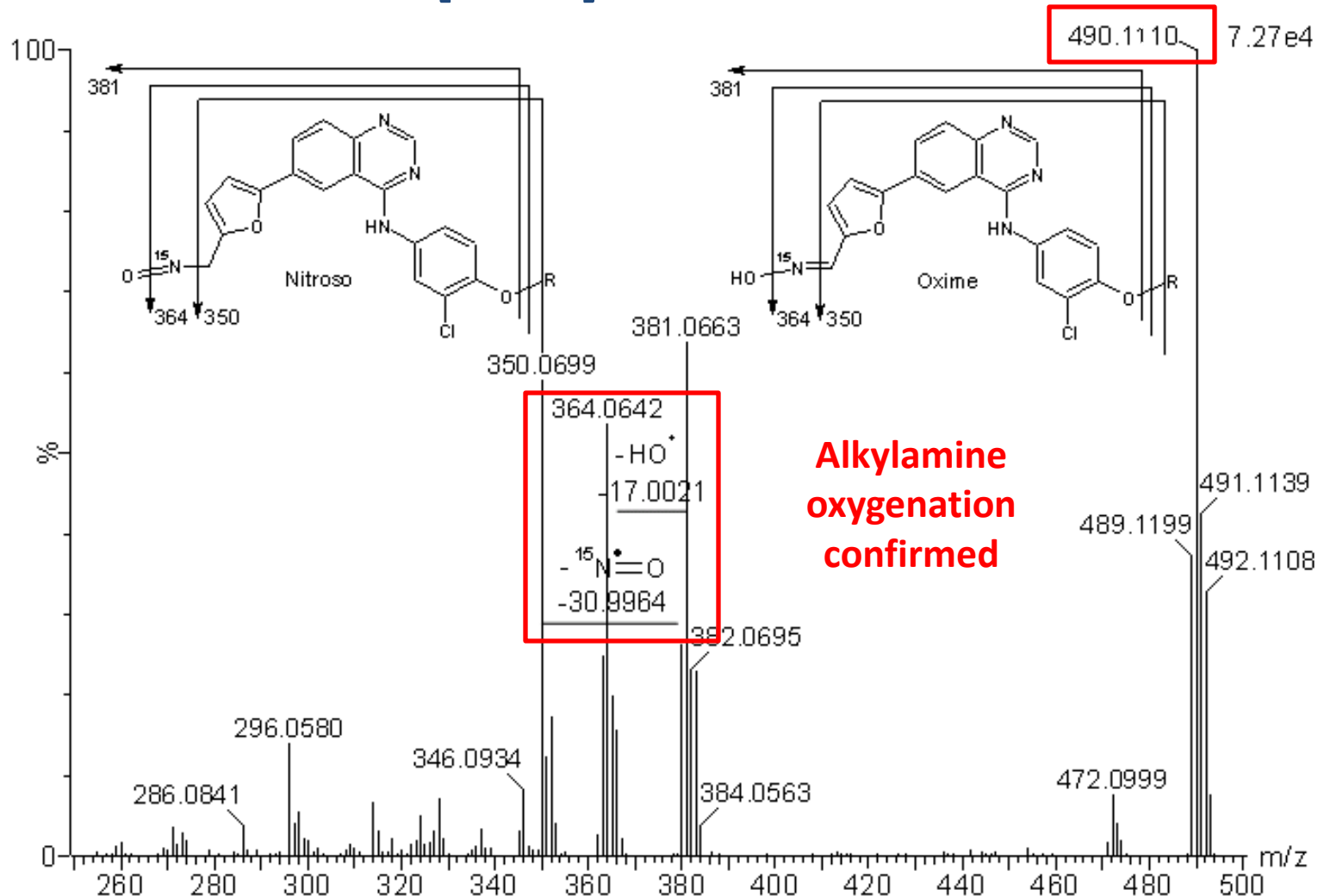
$^{13}\text{C}_2, ^{15}\text{N}$ -Lapatinib

Molecular Formula = $^{13}\text{C}_2\text{C}_{27}\text{H}_{26}\text{ClF}^{15}\text{N}_1\text{N}_3\text{O}_4\text{S}$
Monoisotopic Mass = 583.138476 Da

+3 from unlabeled lapatinib

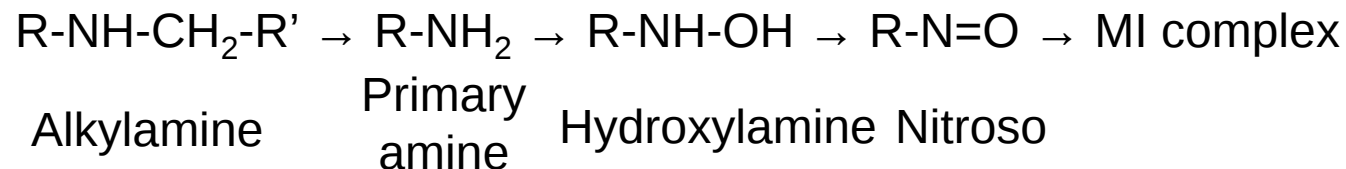
MSMS data for M9 formed from $^{13}\text{C}_2, ^{15}\text{N}$ -lapatinib

- Mass shift of +1 in $[\text{M}+\text{H}]^+$ and NO radical neutral loss



Nitroso formation from secondary alkylamines

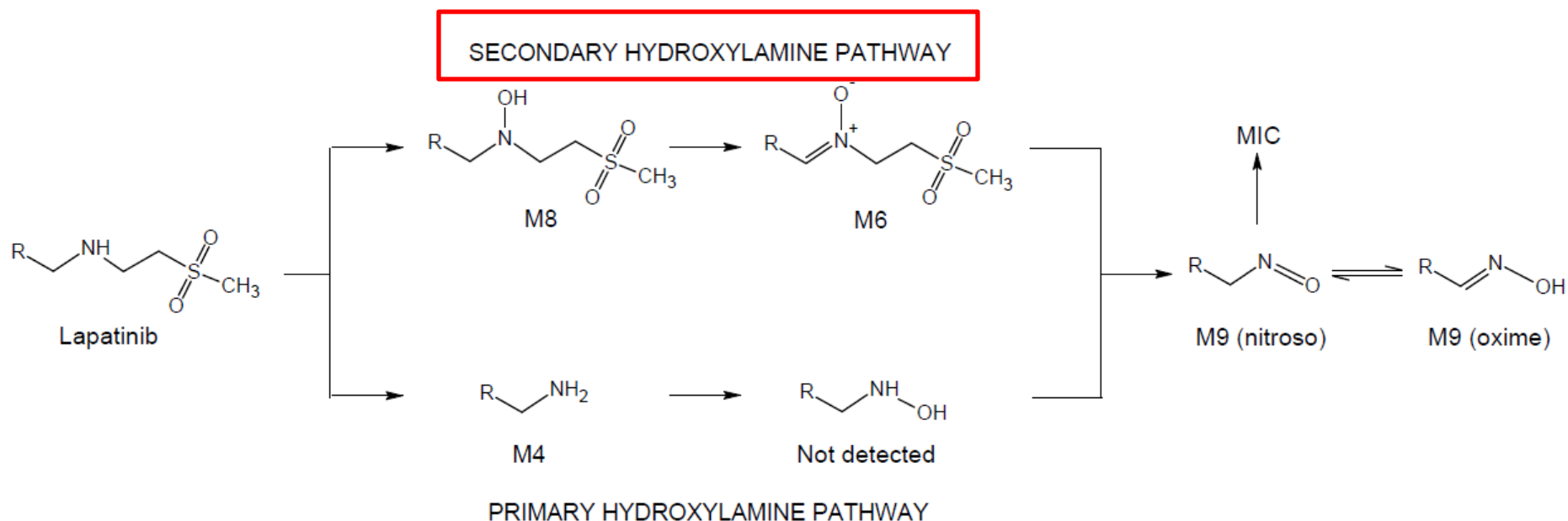
- Traditional pathway to nitroso formation



- In 2010, Hanson *et al.* showed compelling evidence that N-hydroxylation of the secondary amine is the pathway for secondary alkylamines
- They also showed evidence that the N-dealkylation to the primary amine may be a competitive pathway

Hanson et al., (2010) *Drug Metab Dispos* **38**: 963-972.

Secondary hydroxylamine pathway supported



- We detected all of the intermediates in the secondary hydroxylamine pathway
- We did not detect the primary hydroxylamine intermediate in the primary hydroxylamine pathway

Conclusions

- Ultracentrifugation/chemical oxidation with metabolite profiling can be used for mechanistic studies on quasi-irreversible CYP inhibition
- The *N*-desalkyl nitroso (detected as its oxime tautomer) metabolite of lapatinib is responsible for the mechanism-based inhibition of CYP3A4
- Our data supported the secondary hydroxylamine pathway to nitroso formation through the nitron intermediate



Thank you

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