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

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Overview

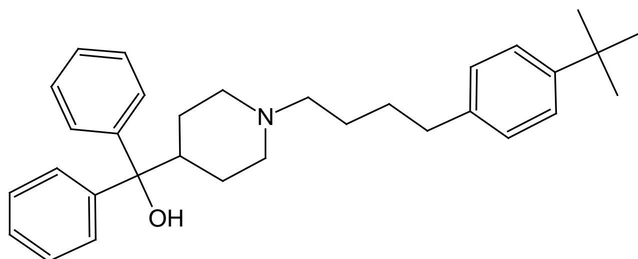
- Cytochrome P450 (CYP) inhibition in drug development
- Metabolite profiling in drug development
  - Questions addressed
  - Accurate mass spectrometry
- Mechanistic studies in enzymatic inactivation caused by lapatinib

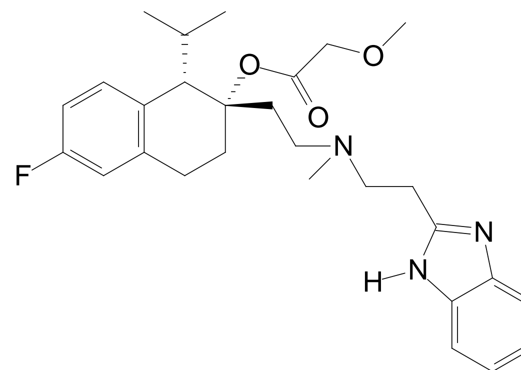
Cytochrome P450 inhibition

- 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Terfenadine: withdrawn 1997 (victim drug)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Mibefradil: withdrawn 1998 (perpetrator drug)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

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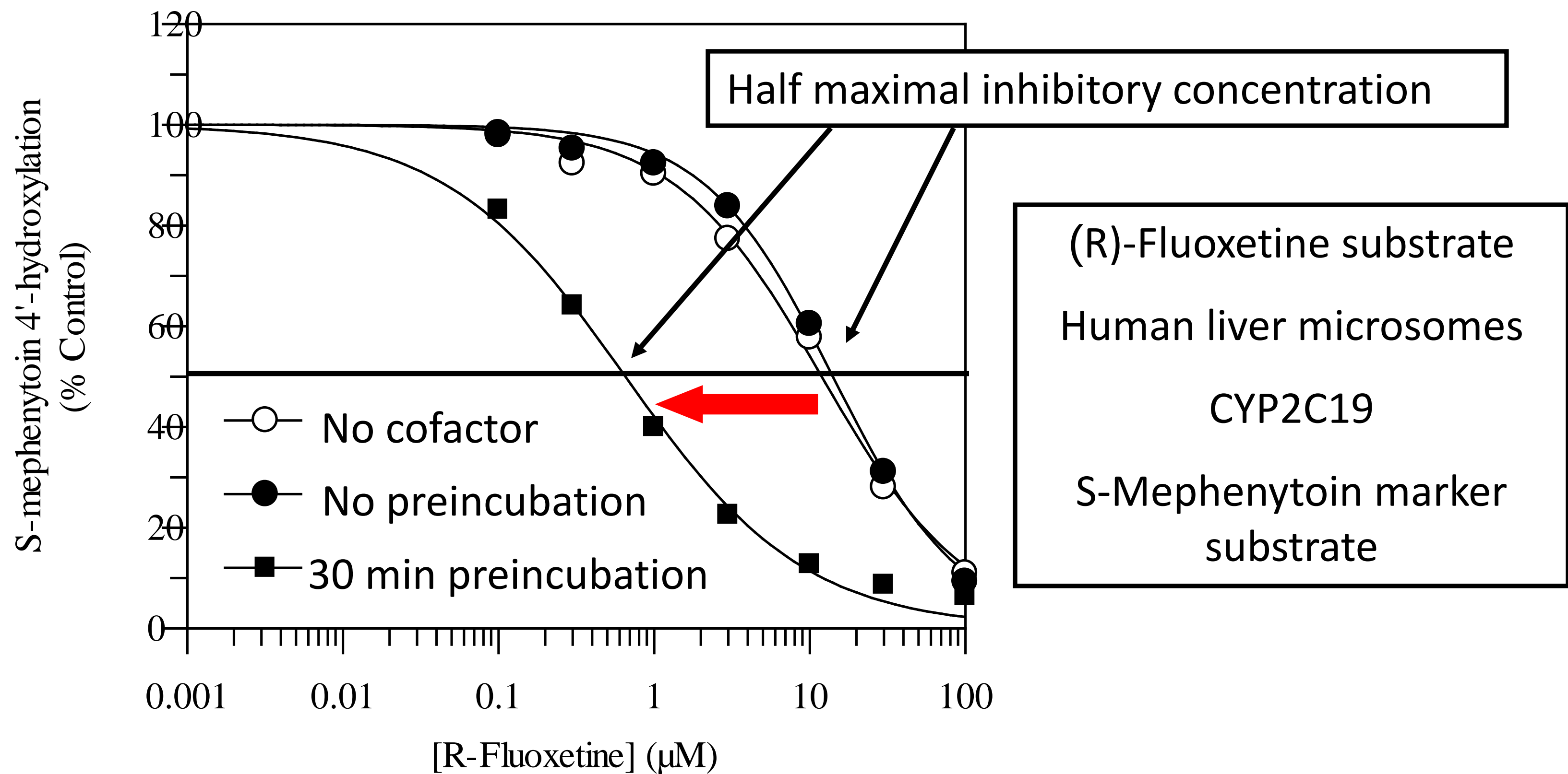
Establishing metabolism-dependent inhibition

- 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



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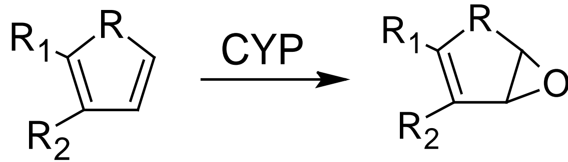
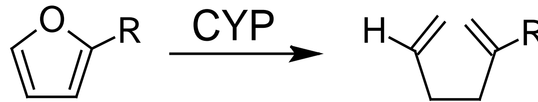
Metabolism-dependent cytochrome P450 inhibition (MDI)

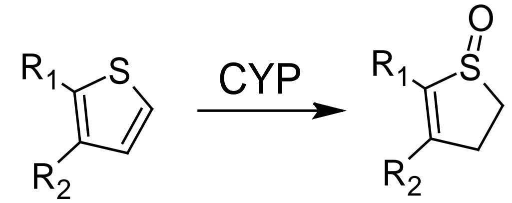
- Three main categories
- Reversible inhibition
  - Metabolite is a potent direct CYP inhibitor
- Irreversible inhibition
  - Metabolite binds covalently to the heme or apoprotein
- Quasi-irreversible inhibition
  - Inhibitory metabolite forms a stable metabolic intermediate (MI) complex with the ferrous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

Functional groups associated with metabolism-dependent inhibition

Several functional groups are associated with metabolism-dependent inhibition



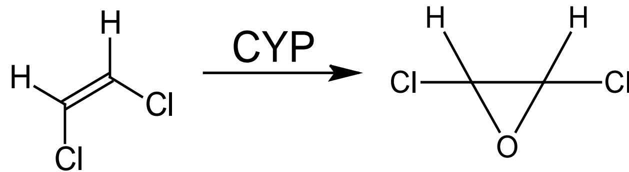




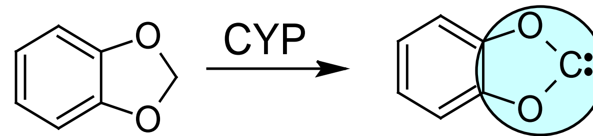
Furans/thiophenes/multiple bonds

Furans/methylfurans

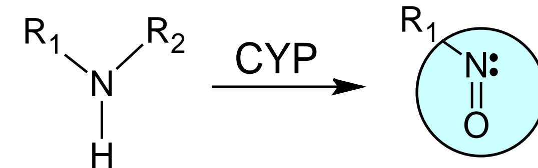
Thiophenes



Di/trichloroethylenes



Benzodioxoles



Alkylamines

But only these two are associated with quasi-irreversible inhibition

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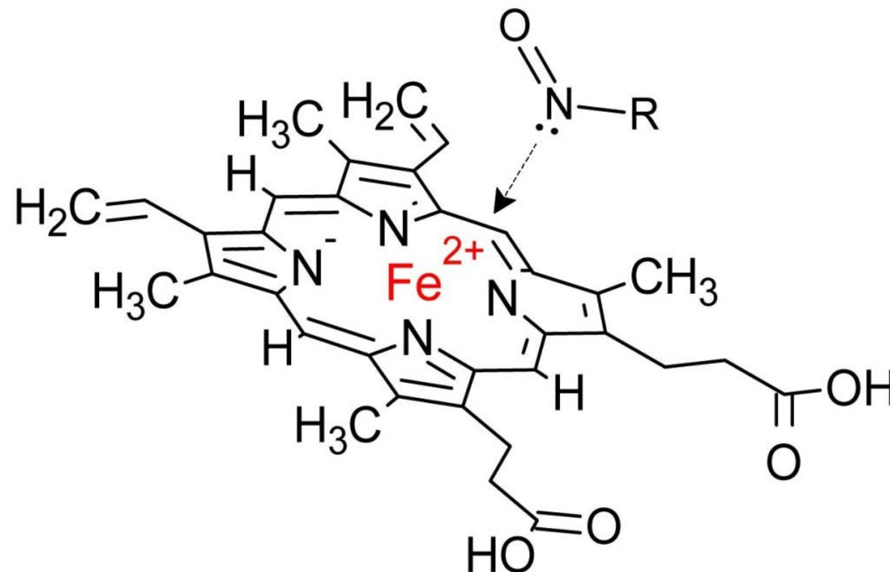
Fontana *et al.* **2005** *Curr. Drug Metab.* **6:** 413-454

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

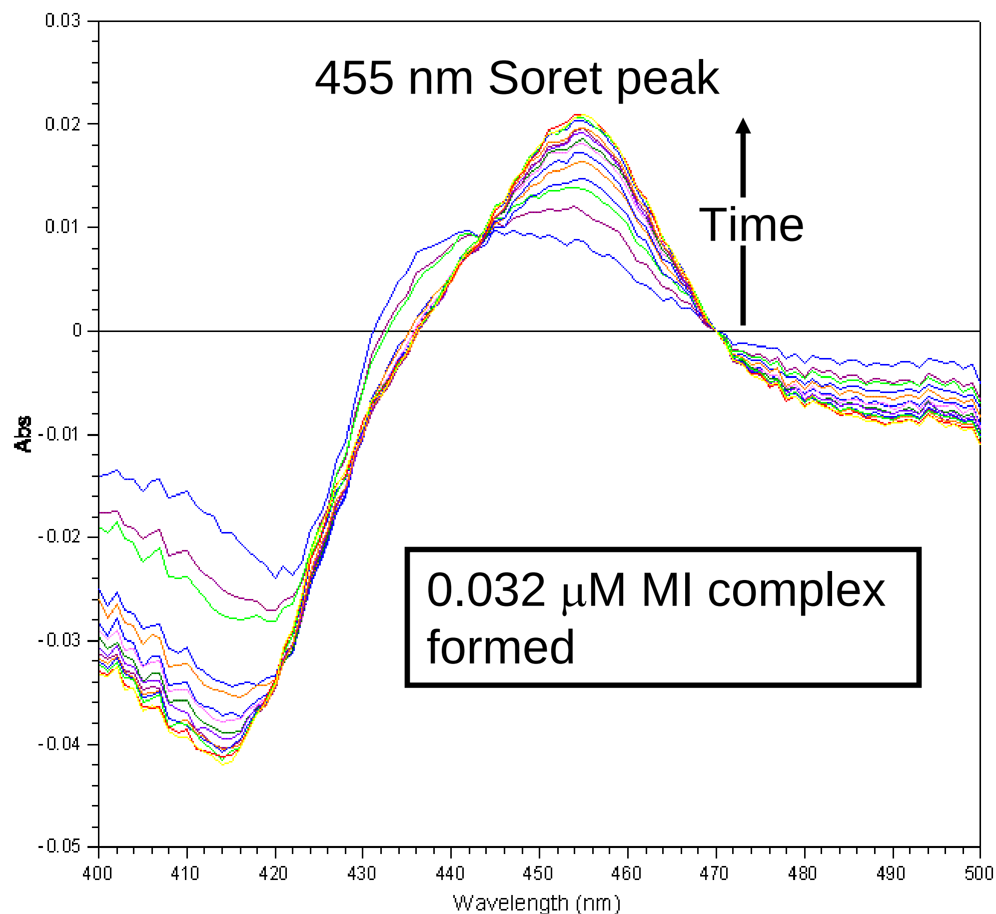


- Can be detected with optical spectrophotometry (λ_{max} ~455 nm)
- Complex can be reversed by oxidizing the iron to the ferric state (Fe^{3+}) with a chemical oxidizer (e.g., ferricyanide)

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



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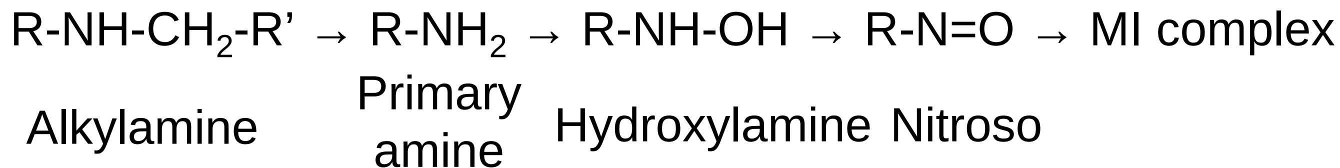
Quasi-irreversible inhibition associated with alkylamines

- Secondary/tertiary alkylamines can cause quasi-irreversible inhibition

NH_3	CH_3-NH_2	CH_3-NH-CH_3	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

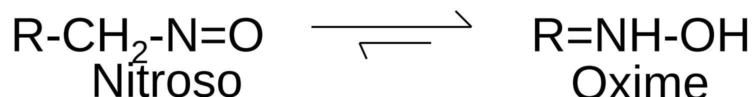
    Danan et al., (1981) *J Pharmacol Exp Ther* **218:** 509-514. Pessayre et al., (1981) *Biochem Pharmacol* **30:** 553-558.

    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.



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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, ^{3}H, ^{35}S)
  - Mass spectrometric detection
- Perform structural elucidation of detected components with mass spectral data

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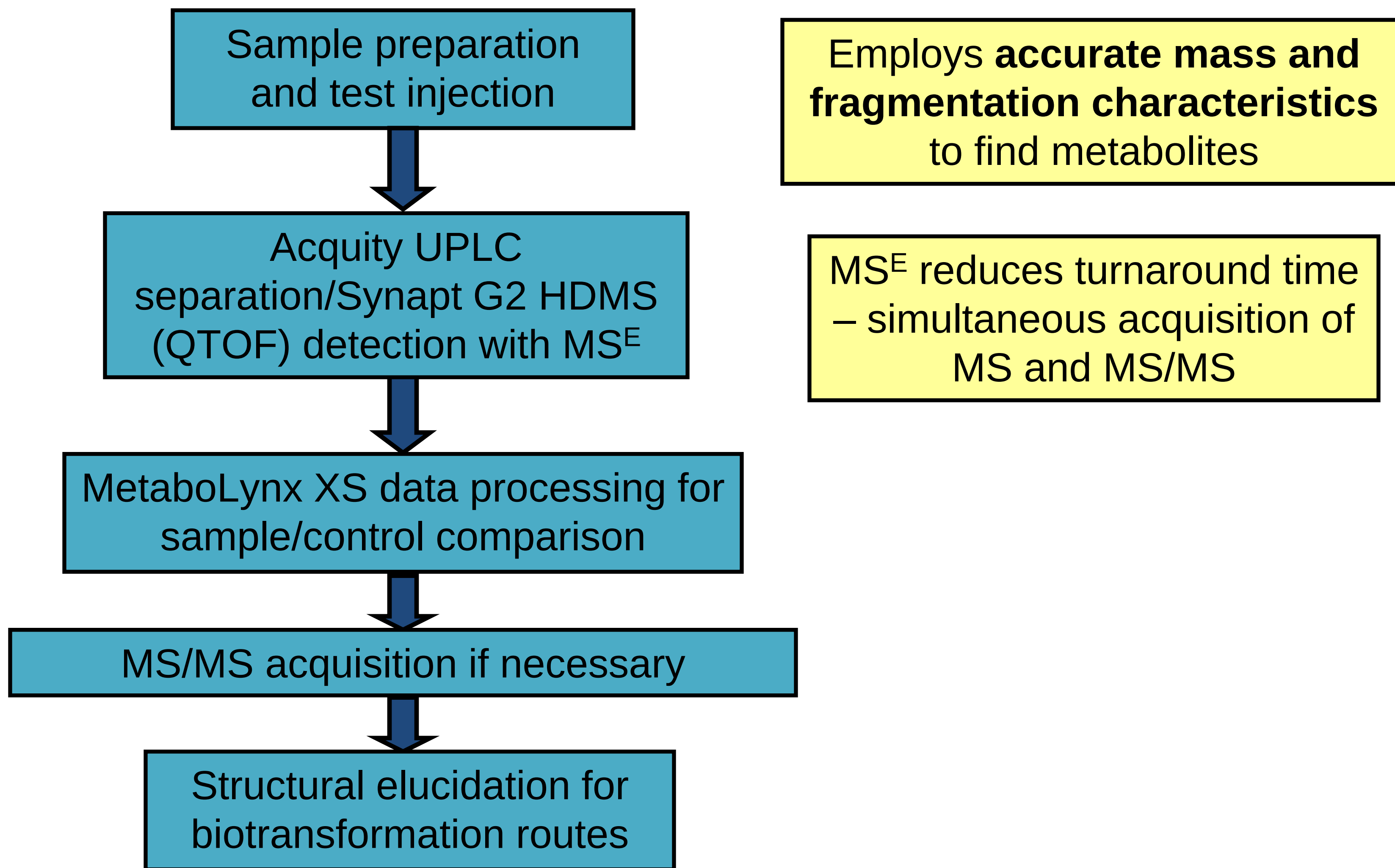
Accurate mass spectrometry for metabolite profiling

- 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



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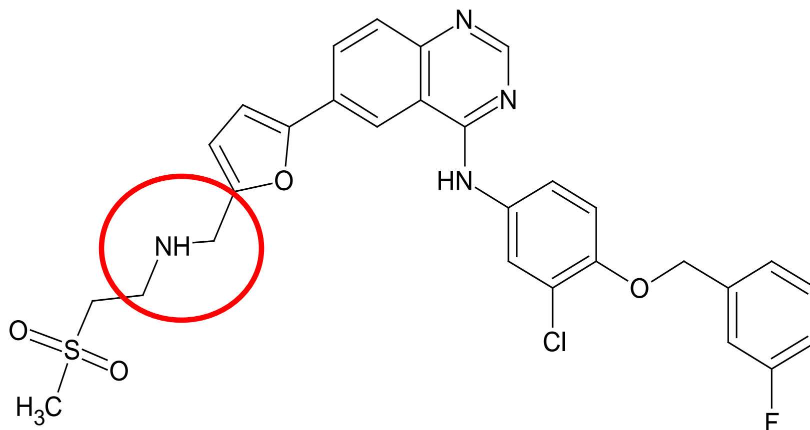
Accurate mass spectrometry benefit

- 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	$+H_2O$	
+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

Takakusa H, Wahlin MD, Zhao C, Hanson KL, New LS, Chan ECY and Nelson SD (2011) *Drug Metab Dispos* **39(6):** 1022-1030.

Hypothesis

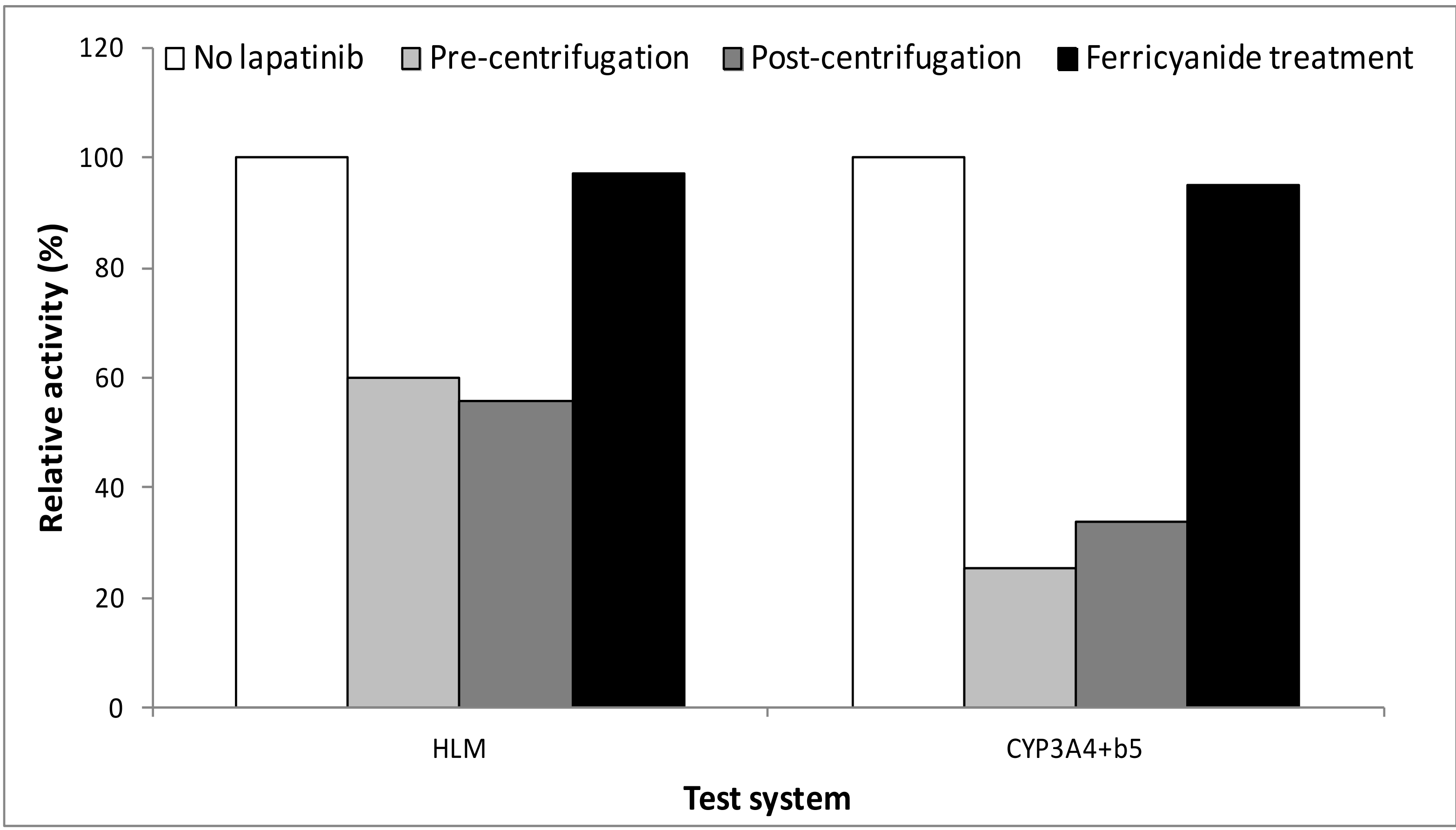
In vitro incubation with ultracentrifugation, chemical oxidation by ferricyanide, and liquid chromatography/tandem mass spectrometry-based metabolite profiling could identify the complexed lapatinib metabolite(s) causing the CYP3A4 inhibition

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

Lapatinib

O-Dealkylation

M1

3A4 / 3A5

O-Dealkylation

M2

3A4 / 3A5

N-Dealkylation

M10

3A4 / 3A5

Reduction

M7

3A4 / 3A5

M4

3A4 / 3A5

N-Hydroxylation

M8

3A4 / 3A5

Oxidation

M6

3A4 / 3A5

OR

M9 (nitroso)

M9 (oxime)

3A4 / 3A5

C-Hydroxylation

M3

3A4 / 3A5

Hydroxylation

M5

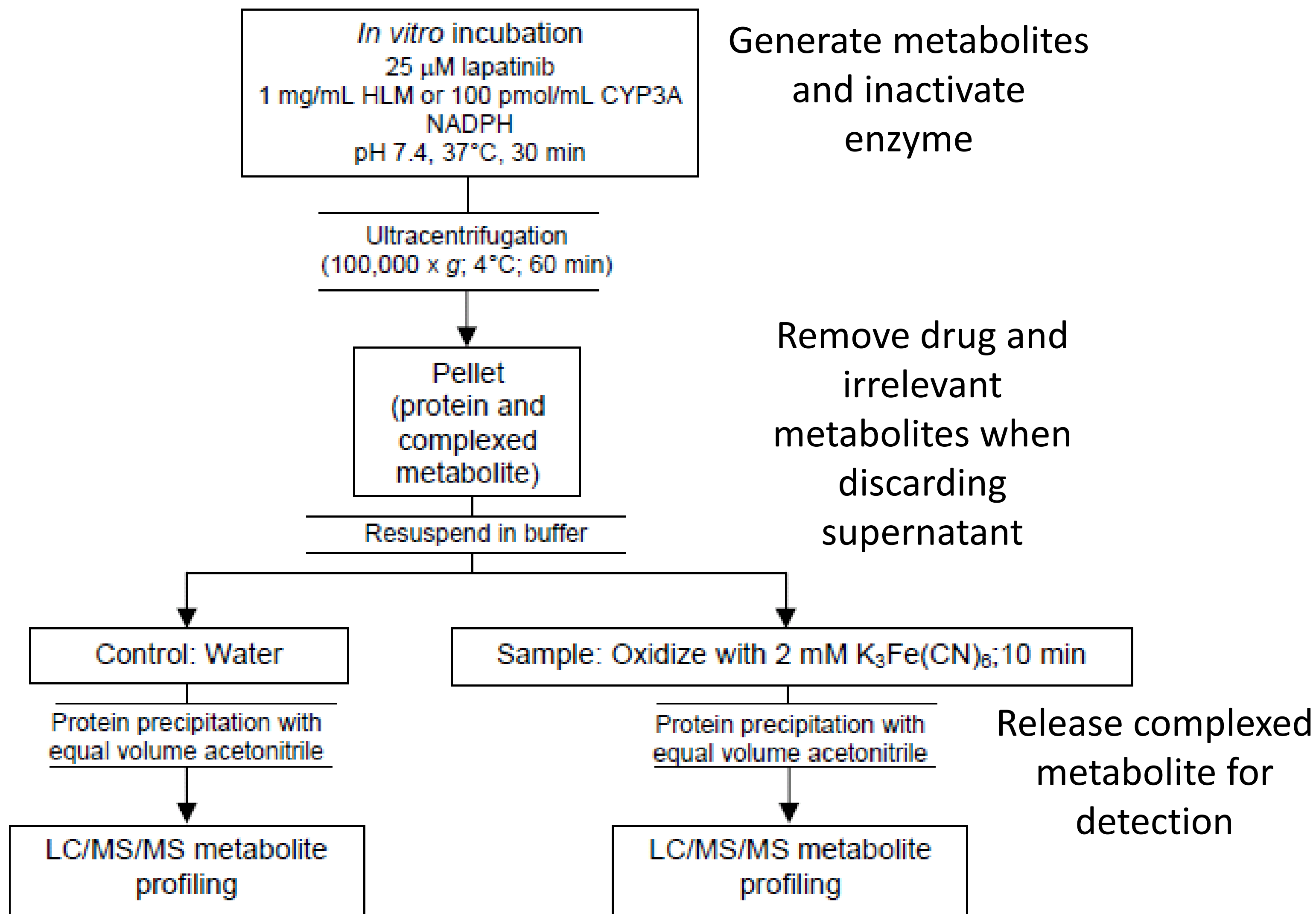
3A4 / 3A5

R =

-(H)

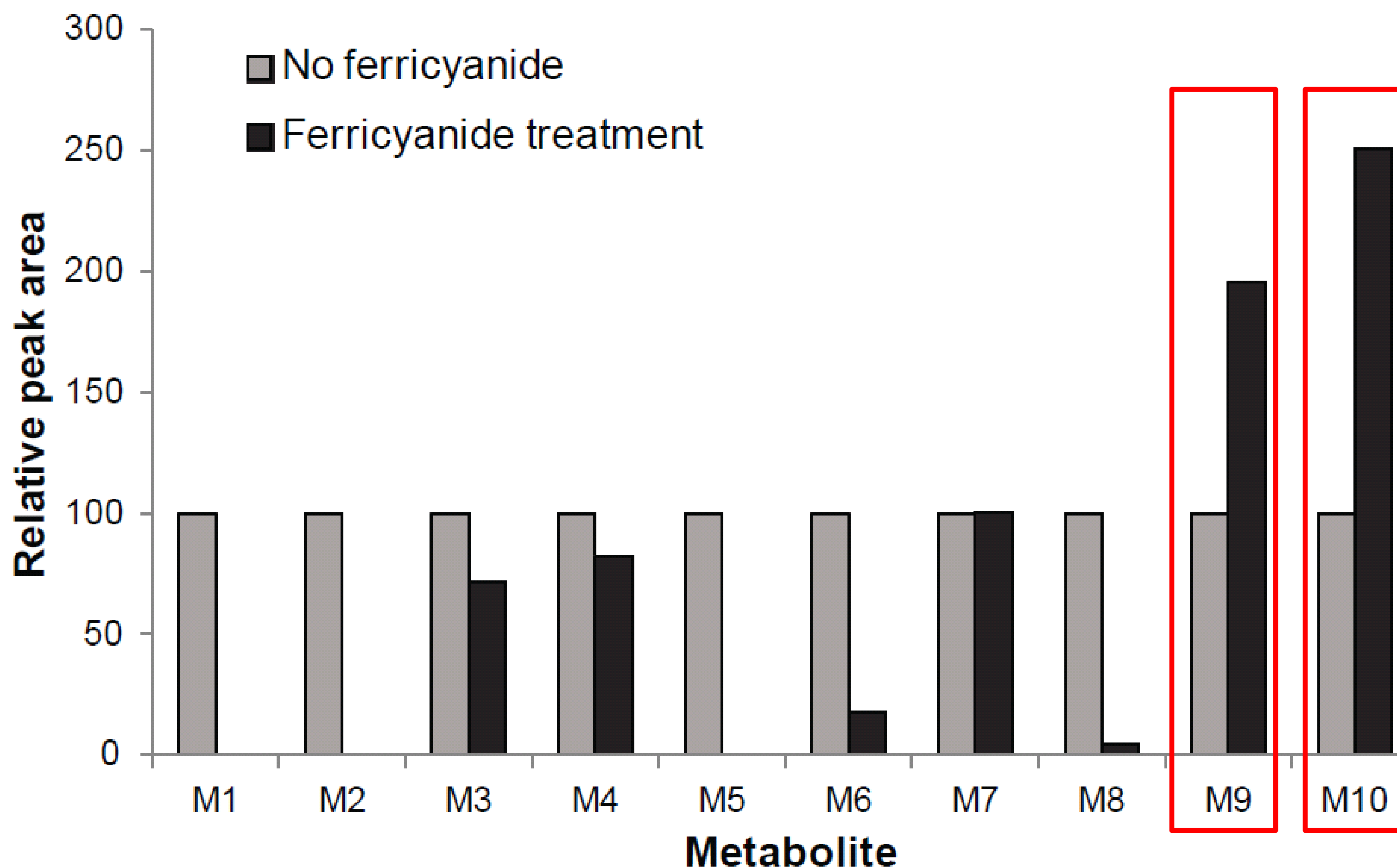
-(H)

Experimental method



Ferricyanide effect on metabolite formation in HLM

- M9 and M10 increased with ferricyanide oxidation



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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.

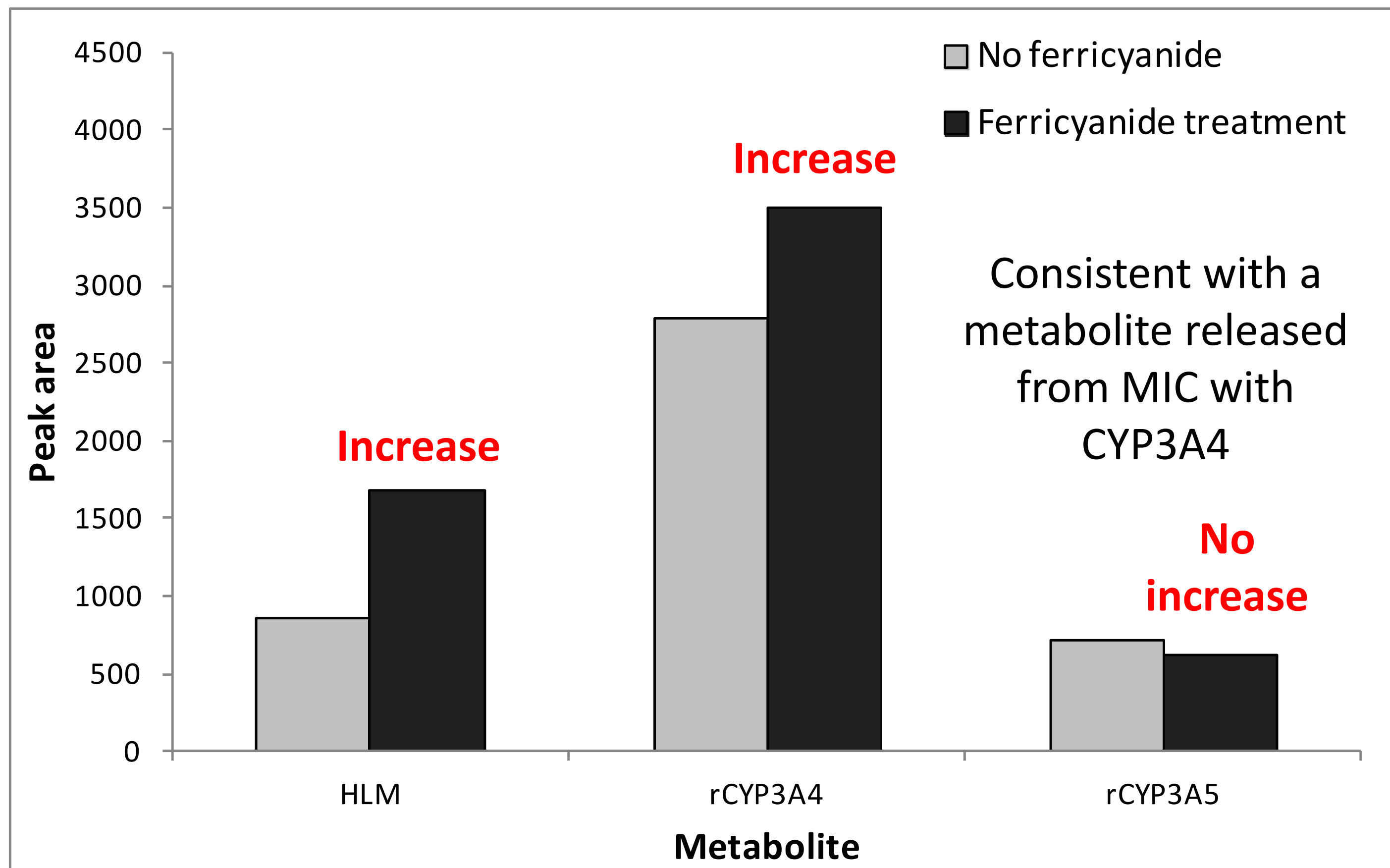
- Lapatinib is irreversible inhibitor of CYP3A5 (primarily O-dealkylation metabolism)

  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



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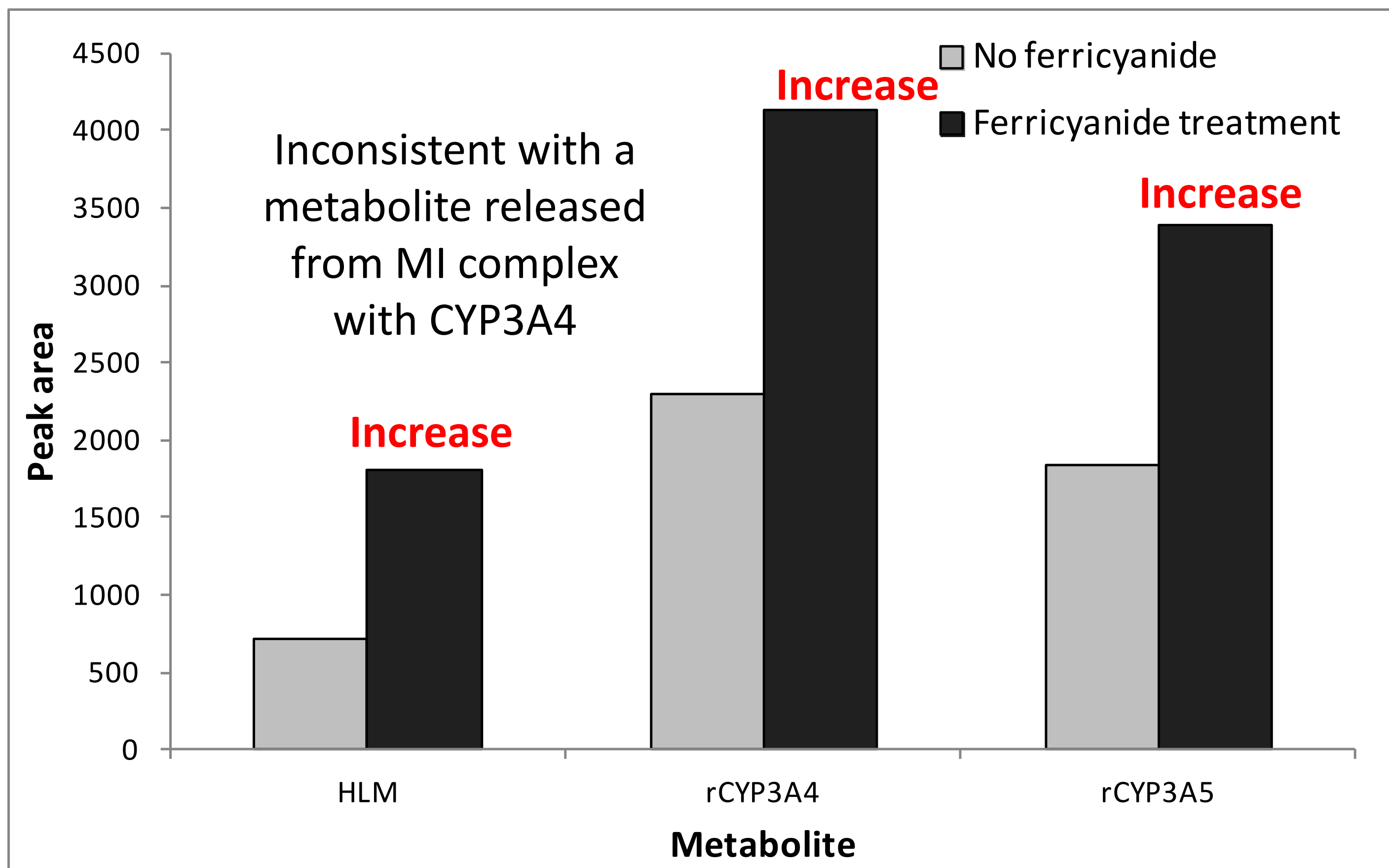
Recombinant CYP3A4 *versus* CYP3A5: M10

- Effects of oxidation show no test system differences



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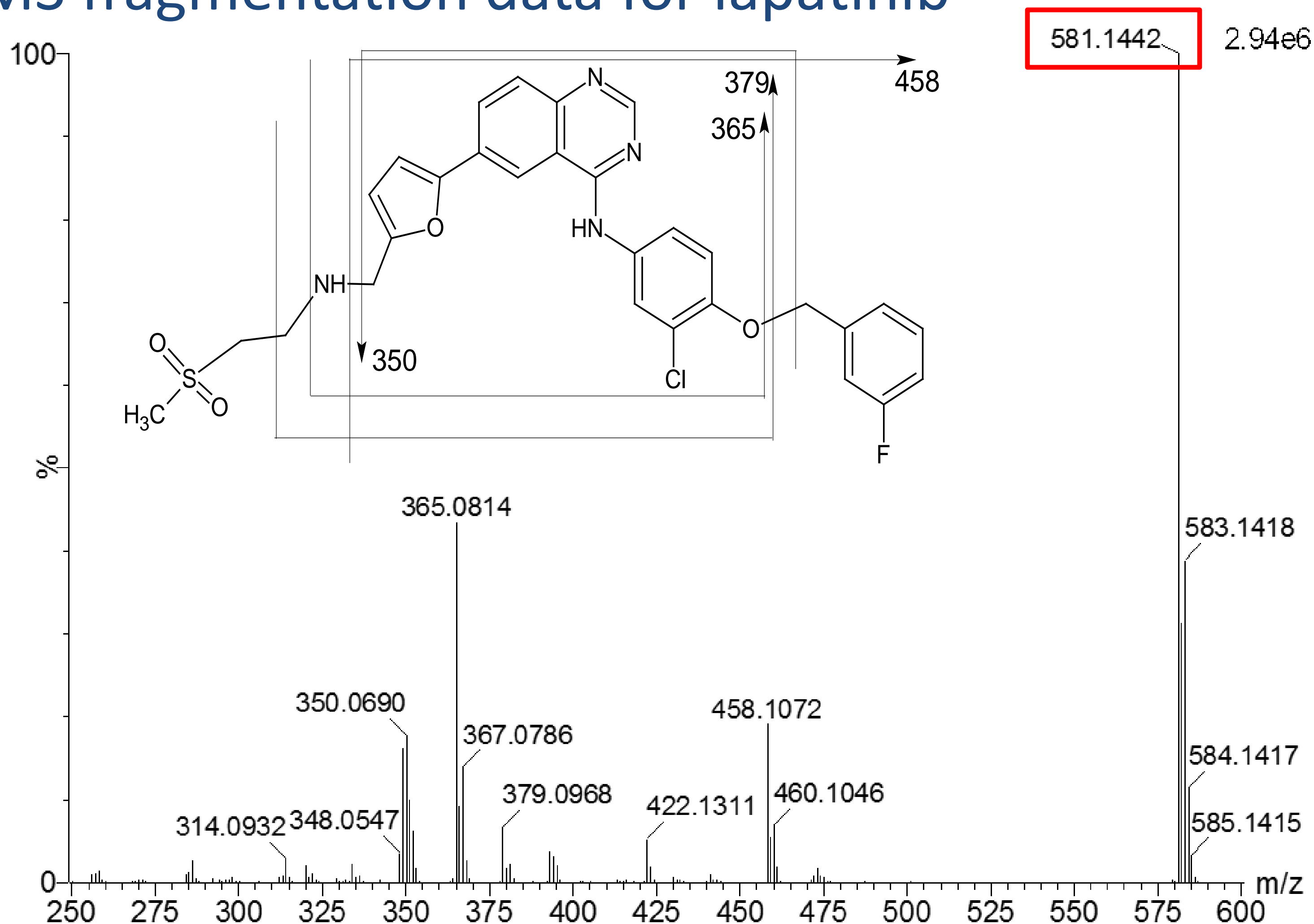
Identifying M9: Structural elucidation

- MSMS fragmentation data for lapatinib



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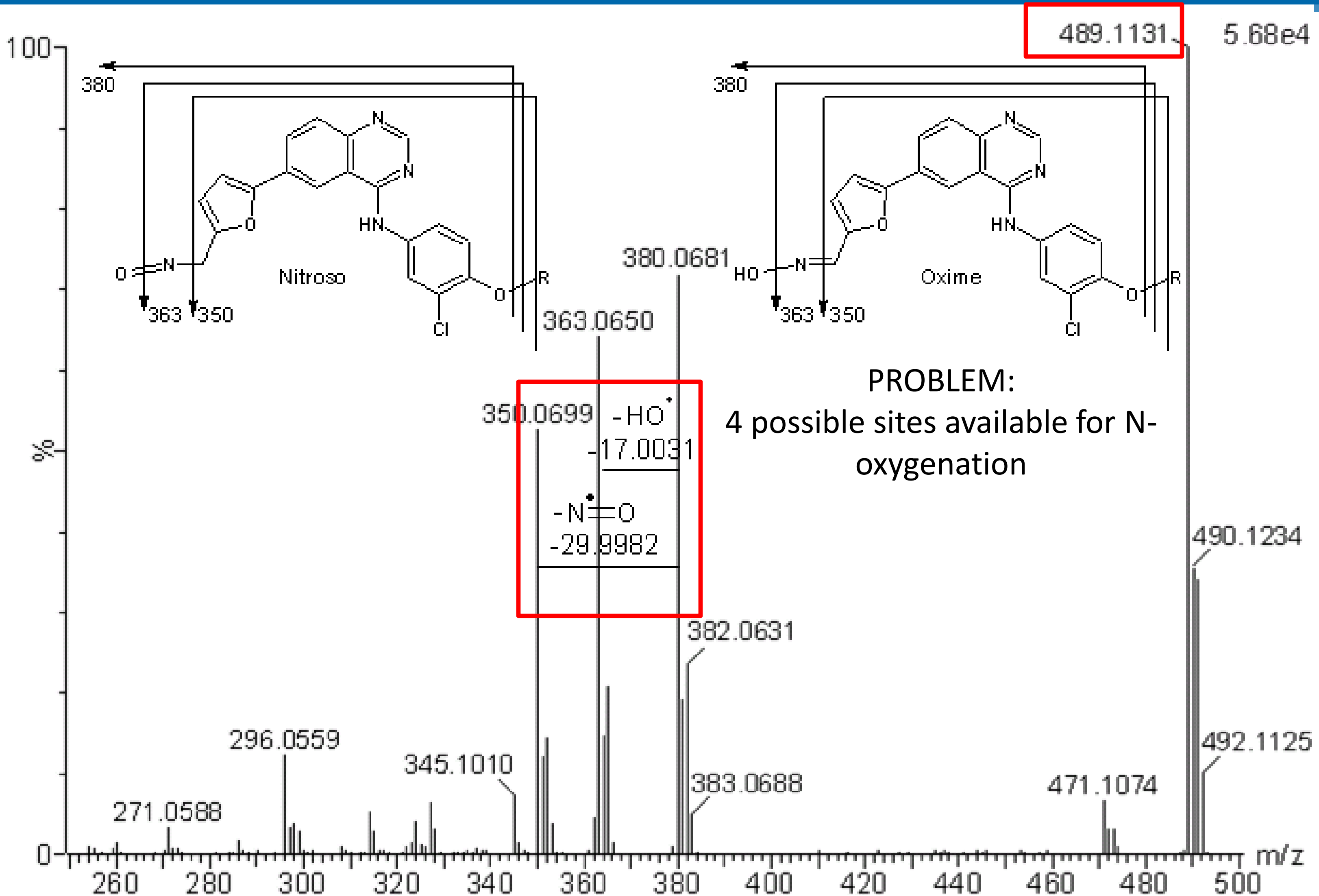
M9 MSMS fragmentation data with proposed assignments



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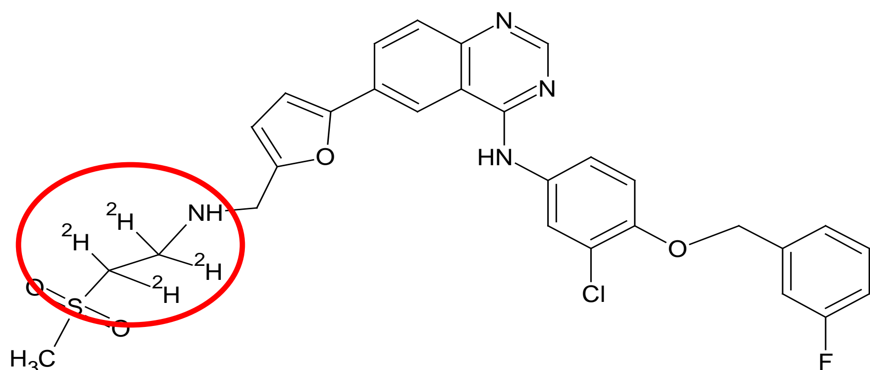
Stable-isotope labeling experiments

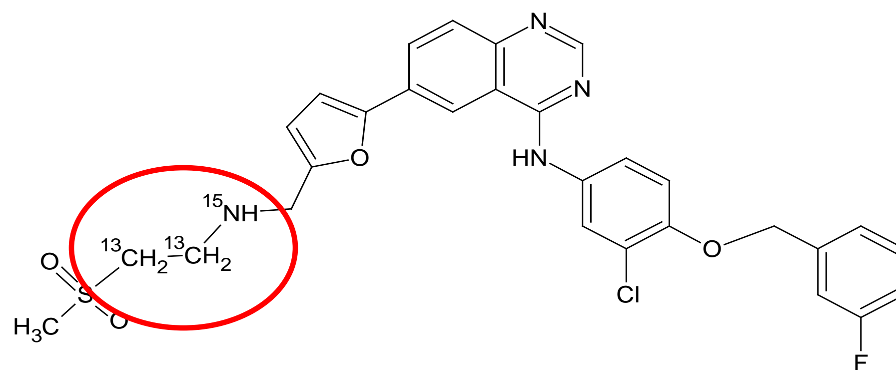
- No metabolite standards available
- $^{2}H_4$-lapatinib and $^{13}C_2$,^{15}N-lapatinib were available



$^{2}H_4$-Lapatinib

Molecular Formula = $C_{29}{}^{2}H_4H_{22}ClFN_4O_4S$
Monoisotopic Mass = 584.159838 Da

+4 from unlabeled lapatinib



$^{13}C_2$^{15}N-Lapatinib

Molecular Formula = $^{13}C_2C_{27}H_{26}ClF^{15}N_1N_3O_4S$
Monoisotopic Mass = 583.138476 Da

+3 from unlabeled lapatinib

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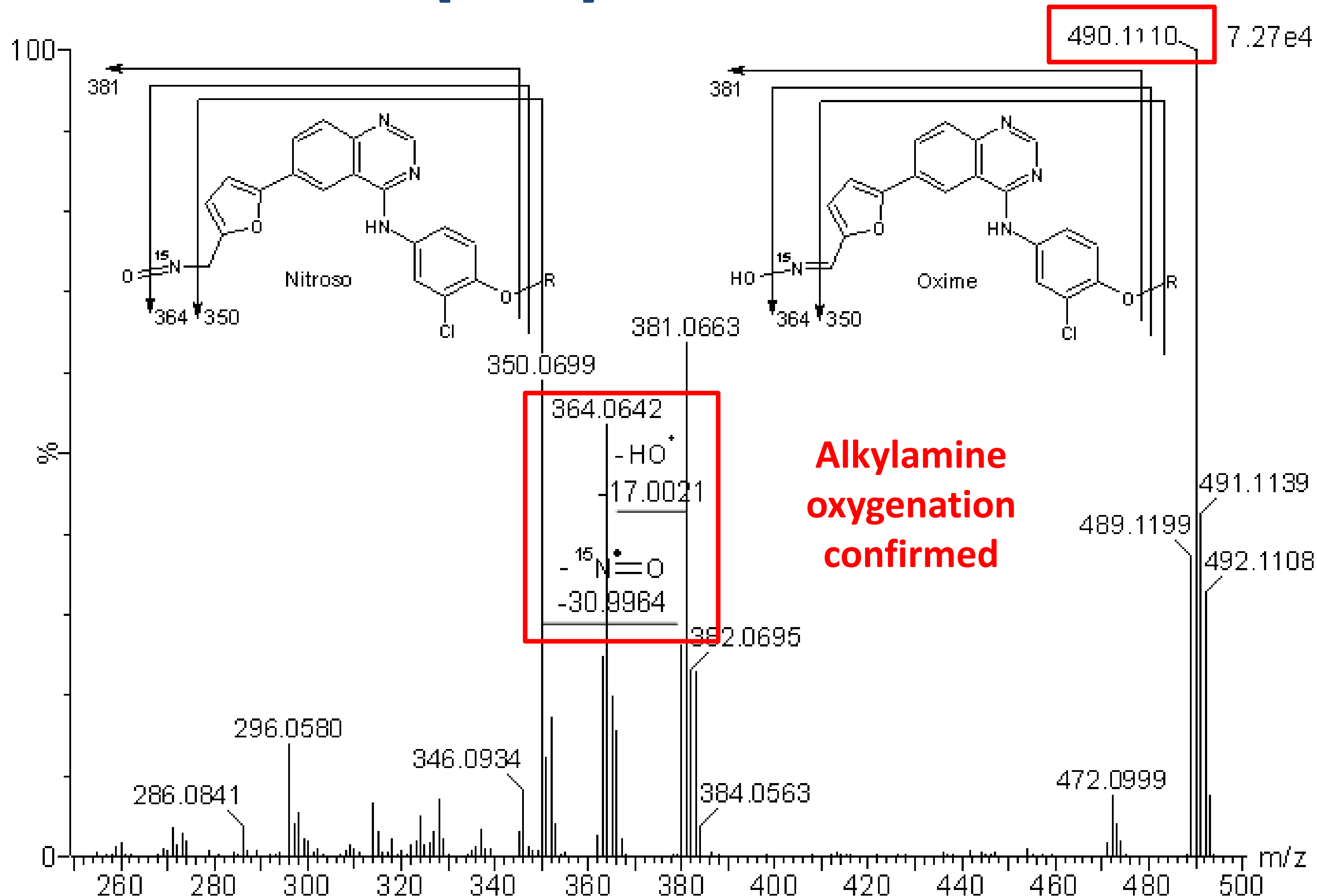
MSMS data for M9 formed from $^{13}C_2,^{15}N$-lapatinib

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



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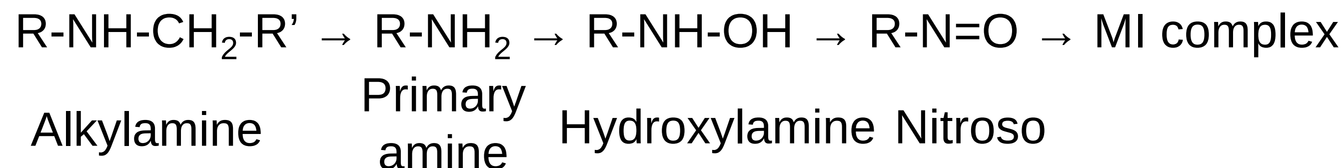
Nitroso formation from secondary alkylamines

- 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.

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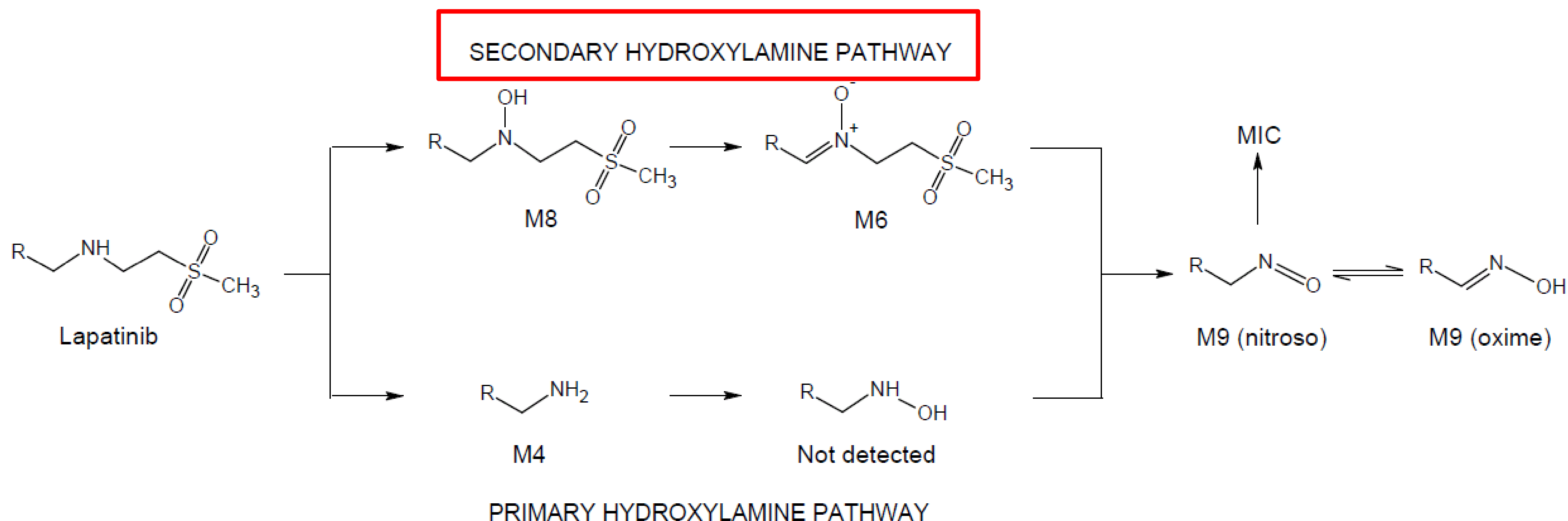
Secondary hydroxylamine pathway supported



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

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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 nitrone intermediate

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Thank you

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