JOURNAL OF NATURAL PRODUCTS

Review

pubs.acs.org/jnp

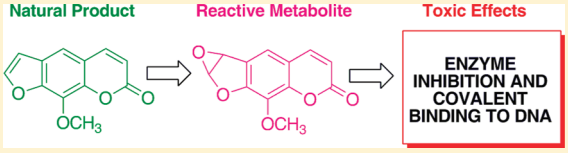
Identification and Characterization of Reactive Metabolites in Natural Products-Driven Drug Discovery

Nicholas M. Njuguna,[†] Collen Masimirembwa,[‡] and Kelly Chibale*[,†]

[†]Department of Chemistry and Institute of Infectious Disease and Molecular Medicine, University of Cape Town, Rondebosch, 7701, South Africa

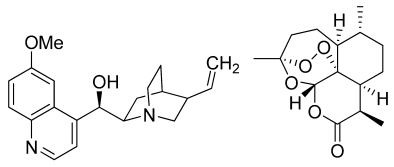
[‡]African Institute of Biomedical Science and Technology, Harare, Zimbabwe

ABSTRACT: Toxicity of natural products arising from their metabolic biotransformation into reactive chemical intermediates is an important reason for high attrition rates in early drug discovery efforts. Screening promising natural products for their likelihood to form such metabolites is therefore an important step in identifying potential liabilities in the drug development process. However, such screening is complicated by the need to have test methods that are sensitive, reliable, accurate, efficient, and cost-effective enough to allow for routine identification and characterization of the reactive metabolites. These metabolites are typically formed in minute quantities, usually through minor metabolic pathways, and, due to their highly reactive and therefore transient chemical nature, pose considerable analytical challenges in attempts to determine their properties. Understanding the formation of reactive metabolites may be used as the basis for synthetic chemical modification of parent natural products aimed at bypassing such harmful bioactivation. This paper highlights the general principles and protocols commonly used to predict and study the formation of reactive metabolites in vitro and how the data obtained from such studies can be used in the development of safer drugs from natural products.



INTRODUCTION

Natural products have, for hundreds of years, provided a primary source of drugs and continue to do so even today.[1] Since morphine became the first pharmacologically active natural compound to be isolated in its pure form in 1805, numerous other molecules have been discovered and used as the basis for clinical treatment or as precursors of semisynthetic analogues.[2,3] This is exemplified by compounds such as quinine (**1**) and artemisinin (**2**), which represent just two of the numerous natural products known to exhibit antiplasmodial activity and that either are currently widely used as first line drugs or have been the starting point for much superior semisynthetic analogues, in the treatment of malaria.[4−6]



1 2

Figure 1. Quinine (**1**) and artemisinin (**2**).

Notwithstanding these natural product-inspired successes, over the last few decades the development of drugs from natural sources has undergone a noticeable decline, with more emphasis being placed on synthetic compounds.[7] Nevertheless, recognition of the fact that the natural environment holds almost limitless and largely unexplored potential sources of compounds possessing pharmacological activity continues to draw interest from researchers. Additionally, the realization that some natural products exhibit physicochemical properties, such as cLogP, that resemble closely those of successful synthetic drug molecules has contributed to the sustained interest in this field.[8,9]

Despite this promising potential, one factor that contributes to the low uptake of both natural products and promising synthetic compounds for further development into drugs for clinical use is their metabolic bioactivation into toxic chemical species.

METABOLISM OF XENOBIOTICS

All foreign compounds, regardless of their nature as nutrients, drugs, food additives, pollutants, industrial chemicals, or toxins are targets for metabolic biotransformation once they enter the body. The overall aim of metabolism is to convert all such compounds into chemicals that can be more readily eliminated from the body.[10] The products of metabolism may be classified into three broad classes, depending on their pharmacological properties. These are (a) inactive metabolites, which possess no pharmacological activity; (b) active metabolites, which may have pharmacological properties of less, equal, or greater magnitude than their parent compounds; and (c) reactive metabolites that are capable of covalently reacting with, and altering, the functional macromolecular structure of endogenous targets in vivo, resulting in unwanted toxic effects.[11]

Special Issue: Special Issue in Honor of Gordon M. Cragg

Received: September 28, 2011
Published: February 1, 2012

In humans, metabolism of xenobiotics is catalyzed by a wide array of enzymes in different organs, of which the most important is the liver, in addition to the gastrointestinal tract and the kidneys, skin, lungs, brain, blood, and nasal mucosa. Metabolism usually occurs in two phases. Phase I metabolic reactions, which include oxidation, reduction, and hydrolysis of xenobiotic substrates, are catalyzed largely by the cytochrome P450 superfamily of enzymes, principally in the liver. Phase II reactions involve conjugation of the products of phase I metabolism (or their unaltered parent compounds) to endogenous hydrophilic molecules such as glucuronic acid, amino acids, and sulfates, or through acetylation. The desired net result of these processes is the conversion of a lipophilic compound into a more polar hydrophilic product that can be more readily excreted from the body.[12−16]

REACTIVE METABOLITES AND TOXICITY

The study of reactive metabolites was pioneered in the 1940s through the work of Elizabeth and James Miller while investigating the metabolism of the carcinogenic azo dye 4-dimethylaminoazobenzene (DAB) (formerly used as a food coloring).[17]

As already mentioned, in the presence of reactive metabolites, the functional macromolecular structure of endogenous targets such as proteins and nucleic acids is altered in vivo.[18] This usually results in profound effects on the normal functioning of the endogenous biomolecules.[19] For example, binding of reactive metabolites to nucleic acids may result in carcinogenicity.[20,21]

Reactive metabolites are classified into two broad categories: electrophiles and free radicals. Electrophiles may act as alkylating or acylating agents and are sometimes alternatively classified as being soft or hard species. The classic soft electrophiles, as shown in Figure 2, include epoxides (**3**), α,β-unsaturated carbonyls (**4**), quinones (**5**), quinone methides (**6**), quinone imines (**7**), isocyanates (**8**), isothiocyanates (**9**), episulfonium (**10**), and aziridinium ions (**11**), while aldehydes (**12**) and iminium ions (**13**) are considered hard electrophiles (Figure 3).[22]

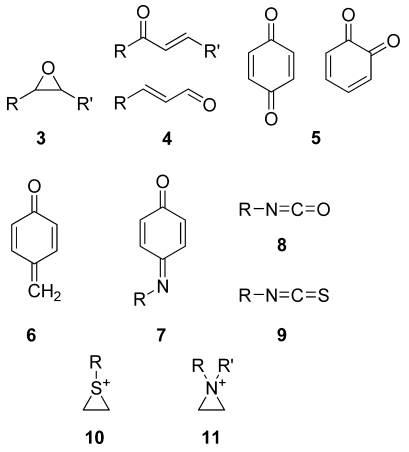


Figure 2. Soft electrophile species.

Electrophiles vary in their selectivity for biological nucleophiles but in general mainly target cysteine, methionine, lysine, histidine, and to a lesser extent glutamate and aspartate amino acid residues on proteins.

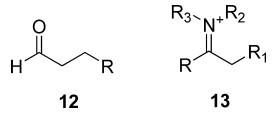


Figure 3. Hard electrophile species.

Free radical reactive metabolites are thought to damage proteins by initiating oxidative stress, resulting in the formation of carbonyl groups. Additionally, free radicals can attack nucleic acids and also target the polyunsaturated fatty acid side chains present in phospholipid bilayer membranes of aerobic cells and tissues.[23]

Although in most cases reactive metabolites are formed from phase I metabolic reactions, they may also arise from the enzymatic conjugation of substrates during phase II conjugation reactions.[24] Covalent binding of reactive metabolite species to intracellular macromolecules is now generally accepted as a key cause of drug toxicity and is believed to play a key role in causing idiosyncratic adverse drug reactions. Most of these reactions are immune-mediated and result from exposure of the immune system to antigens. The vast majority of drugs and their metabolites are typically small compounds with molecular weights below the 1000 Da limit required to elicit such an immune response. However, it has been hypothesized that these compounds may act as haptens and that formation of reactive metabolites and their subsequent binding to produce protein adducts may lead to the creation of compounds of sufficient molecular mass to trigger an immune response typically manifested as type B adverse drug reactions.[25] It should be noted, however, that although binding of reactive metabolites to proteins often results in the functional inactivation of the latter, not all covalent binding automatically results in cytotoxicity.[26−28]

Another mechanism through which reactive metabolites may lead to toxicity is the inhibition of enzymes responsible for the detoxification and elimination of co-administered xenobiotics. It is through this mechanism-based inhibition that many conventional drugs cause drug−drug interactions by forming metabolites capable of covalently binding to, and ultimately inactivating, enzymes that metabolize co-administered drugs. This phenomenon is used as a basis for flagging drug candidates found to inhibit CYP3A4 enzymes during drug discovery to exclude them from further development.[29]

Reactive metabolites from many conventional drugs, and their involvement in causing toxicity and/or adverse drug reactions has been extensively studied and reported, including those from acetaminophen (**14**), valproate (**15**), and diclofenac (**16**), as depicted in Figure 4, as well as from carbamazepine, ketoconazole, and amodiaquine.[30−35]

REACTIVE METABOLITES FROM NATURAL PRODUCTS

Toxicity arising from exposure to some plants may be attributed to the metabolic biotransformation of some of their chemical constituents into reactive intermediates. For example, the formation of reactive metabolites from safrole (**17**), pulegone (**18**), aristolochic acid (**19**), methysticin (**20**), and xanthohumol (**21**), shown in Figure 5 and present in some botanical dietary supplements, has been reported.[36] This is despite the fact that such formulations are generally perceived to be safer than conventional prescription medicines due to their "natural" origins.

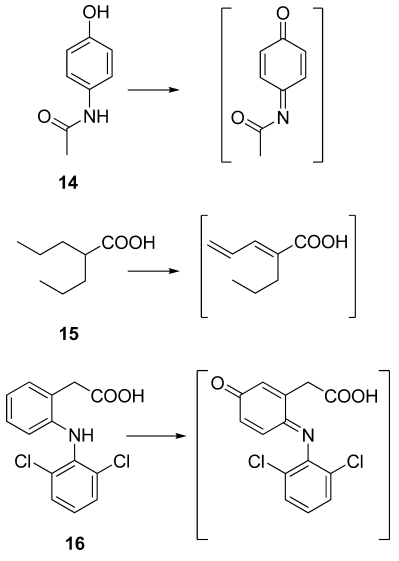


Figure 4. Formation of reactive metabolites from selected conventional drugs.

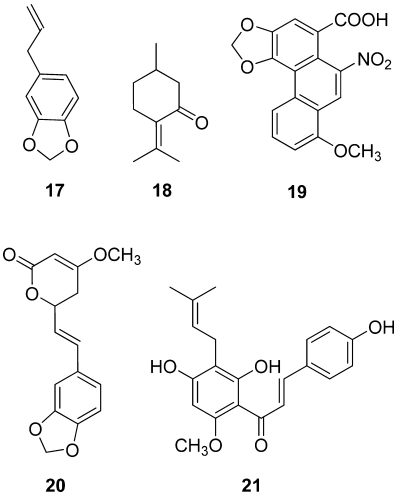


Figure 5. Natural products from dietary supplements metabolized into reactive intermediates.

Additionally, numerous other natural products that undergo similar harmful bioactivation have been reported and studied, ranging from relatively simple structures such as *p*-cresol (**22**) and 4-ipomeanol (**23**) to more complex molecules such as teucrin A (**24**), as depicted in Figure 6.[37−39]

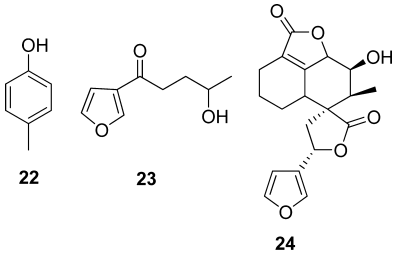


Figure 6. Examples of the diverse structural complexity of natural products.

Predicting Formation of Reactive Metabolites from Structural Alerts. Biotransformation of a compound into reactive metabolites is largely a function of its chemical properties. The presence of certain chemical functional groups has been associated with toxicity due to the formation of reactive metabolites. Compounds containing groups such as acetylenes (**25**), furans (**26**), thiophenes (**27**), benzodioxoles (**28**), anilines (**29**), anilides (**30**), hydrazines (**31**), and hydrazides (**32**), as well as terminal alkenes (**33**), secondary amines (**34**), and conjugated systems (**35**), as shown in Figure 7, have been commonly associated with reactive metabolite-mediated adverse effects.[40−42]

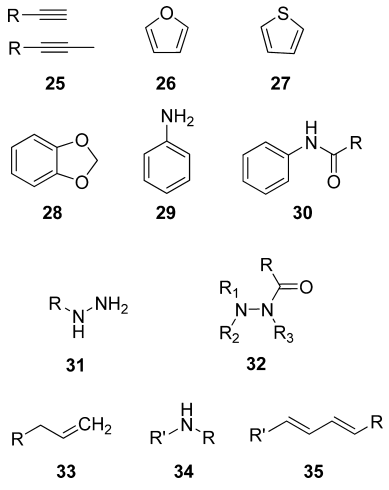


Figure 7. Common chemical structural alert groups.

This awareness can be used in early drug discovery to alert researchers to the possibility of a given compound becoming a metabolic liability. More importantly, once the alert is confirmed, chemical modifications to the structure, such as bioisosteric replacements, that mitigate and/or avoid their formation can be carried out.

In Silico Metabolism Prediction Tools. In silico methods are now used as integral tools in the drug discovery process through the creation of computational models that help to predict such aspects as cell signaling and signal-response behavior, clinical outcomes, and ultimately in identifying drug targets as well as toxicity to different organs.[43,44] In the same way, computational approaches have been developed in predicting CYP-related metabolism properties in screening potential drug compounds.[45] A wide range of approaches and algorithms are incorporated in metabolism prediction software, with some using the structural features and physicochemical properties of test substrate compounds to predict the most likely metabolic sites. Other prediction programs are based on studying homologous substrate−enzyme complexes to determine the structure of the enzyme and, thus, the metabolites likely to arise from biotransformation of the substrate.[46] A third approach involves predicting potential metabolic sites using software that correlates both the physicochemical properties of the drug compound and known substrate−enzyme complexes.[47,48] The latter two approaches are dependent on precise and accurate understanding of the three-dimensional structure of the metabolic enzymes involved in bioactivation of substrates and have gained prominence with the growing use of X-ray crystallography in elucidating such 3-D properties.[49]

Apart from only predicting the possibility of particular metabolites resulting from the biotransformation of a molecule, some software programs are designed to calculate the

dx.doi.org/10.1021/np200786j | J. Nat. Prod. 2012, 75, 507−513

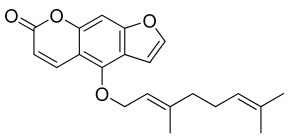
occurrence ratio of products arising from the different metabolic reactions a single compound may undergo.[50] Using this approach, the probability of a compound to be metabolized into reactive intermediates may be predicted in advance with most such software also having the ability to calculate the reaction energies and stability of products from different reaction pathways.

Yet another approach to in silico prediction is the use of knowledge-based database systems that are capable of predicting toxicity of novel compounds based on their similarity to pre-existing substrates for which toxicity profiles have been well characterized. This approach is especially useful due to the existence of large-volume commercial and publicly available databases containing data sets on numerous substrates, their known toxic effects, target organs, metabolizing enzymes, and biotransformation pathways, to which the prediction software may be linked in processing a new compound.[51,52]

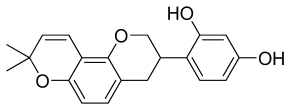
Studying Reactive Metabolites in Vitro. By far the most common technique in early drug discovery for routine in vitro reactive metabolite studies involves the incubation of test compounds in metabolic assay setups to which appropriate trapping agents are incorporated. The basic requirements for such a setup include the following:[53,54]

1. The test compound for which the metabolism to reactive species is under investigation.
2. The source of enzymes required to metabolize the compound. Human liver microsomes are most commonly used. Alternative metabolism vectors to liver microsomes include liver cytosol, liver S-9 fractions, hepatocytes, or even neutrophils. In other cases, recombinant human CYP enzymes expressed from insect cells in whose DNA genes for CYP formation have been transfected may be used. Also reported in the literature is the use of mutants of cytochrome P450 BM3 (CYP102A1) obtained from *Bacillus megaterium* and found to possess more potent biocatalytic activity compared to human liver microsomes and therefore ideal for use in synthesizing large quantities of reactive metabolites.[55]
3. Cofactors required for the enzyme-catalyzed oxidation of the substrate. Since most studies focus on metabolites resulting from oxidation of the xenobiotic substrate by CYP450 enzymes, the cofactor typically incorporated to supply the energy required for the reaction is NADPH.[56] Alternatively, an NADPH regenerating system comprising β-NADP, glucose 6-phosphate, and glucose 6-phosphate dehydrogenase may be used.
4. A trapping agent to "capture" the short-lived reactive metabolites formed in vitro to allow for their detection and characterization. Trapping agents act as targets to which the reactive metabolites generated during the assay bind covalently to form stable adducts that can then be subjected to detection techniques. Commonly used for this are glutathione and its derivatives *N*-acetylcysteine and γ-glutamylcysteinyllysine, methoxylamine, potassium cyanide, and semicarbazide.[57] Dansylated, ethyl-esterified, quaternary ammonium and bromine-substituted glutathione have also been used as alternative trapping agents.[58] Recently, the use of synthetic peptide trapping agents has also been reported.[59]

In the absence of suitable trapping agents, an early indication of the formation of reactive metabolites in vitro may be deduced from evidence of time-dependent enzyme inhibition when test natural products are incubated together with known enzyme substrate controls. Inhibition of enzyme activity in such a setup may be a strong indicator of the natural product being metabolized into reactive chemical species that act as mechanism-based or suicide inhibitors of the metabolizing enzyme by binding irreversibly to it as soon as they are formed. It is through such a mechanism that many compounds that induce drug−drug interactions exert unwanted effects. The inhibition of CYP3A4 by the natural products bergamottin (**36**) from grape fruit juice and parsley (Figure 9) and glabridin (**37**) from licorice are well-established examples of this process.[42,60,61]



36



37

Figure 8. Natural products implicated in mechanism-based enzyme inhibition.

A key advantage of in vitro enzyme inhibition studies is that they facilitate the detection of reactive metabolites that may not be captured using trapping agents because such species bind directly to the enzyme target at the catalytic pocket and therefore cannot be accessed by the trapping agent.

Alternatives to using biological systems to metabolize test compounds into reactive species exist. For example, CYP450 oxidation of substrates mimicked using electrochemical and electrochemically assisted Fenton chemistry as well as synthetic metalloporphines have been reported.[62,63] Such synthetic methods are advantageous in providing easier possibilities for scaling up metabolite synthesis for purposes of carrying out more comprehensive studies on the metabolites thus generated.

Regardless of whether or not reactive metabolites are synthesized using a biological or electrochemical system and subsequently covalently bound to the trapping agent, LC-MS techniques are almost universally used as the pre-eminent choice for their detection and identification. Development and use of tandem mass spectrometers equipped with such components as linear ion or quadrupole linear ion traps and quadrupole time-of-flight and orbitrap mass analyzers has greatly increased the sensitivity of reactive metabolite detection.[64,65]

■ CURRENT CHALLENGES

The most challenging aspect of in vitro reactive metabolite studies lies in developing and validating techniques that are sensitive enough to detect the minute quantities of the reactive intermediates generated experimentally. No single trapping agent has been identified that can be used to detect the different species of reactive metabolites possible. As a result, the danger of in vitro experiments returning false negative results due to the use of an inappropriate trapping agent remains a real impediment when evaluating experimental data. For example, glutathione may be appropriate for trapping epoxide reactive

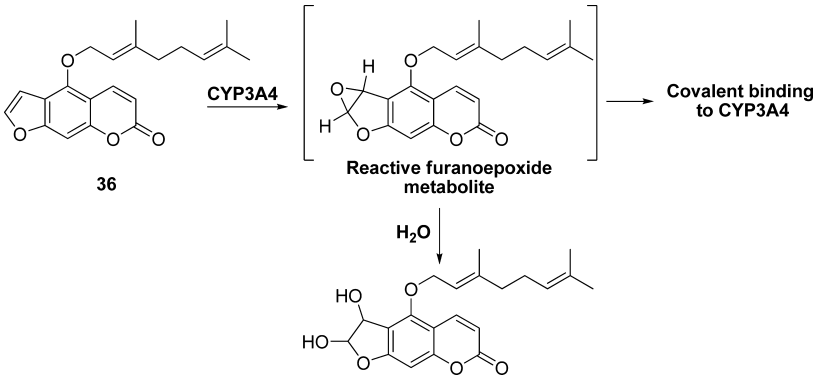


Figure 9. Scheme summarizing proposed bioactivation of bergamottin (**36**).[60]

metabolites but unsuitable for screening free radicals.[66] To address this, novel trapping agents capable of forming adducts with such compounds that lower their analytical detection limits are continually being sought after and reported in the literature, as indicated previously.[58,59]

Although most xenobiotic biotransformation reactions are catalyzed by CYP450 enzymes, false negative in vitro trapping experiments may arise from the evaluation of compounds that are metabolized into reactive species through non-CYP-catalyzed oxidation reactions. These therefore would not be detected using experimental setups using only microsomes as the source of enzymes.

Another challenge lies in the software used to predict the propensity of a compound to be metabolized into one or more reactive species. Most commercially available software for such predictions are designed to forecast metabolism using training sets based on rules developed from models of the known physicochemical properties of mainly synthetic or semisynthetic commercial drug molecules. Whereas this might be appropriate for predicting the biotransformation of synthetic molecules, using the same models to forecast the metabolism of natural products may not be as accurate, especially since these compounds often occupy a different and unique chemical "universe" from that of most commercially synthesized drug molecules.

Most studies tend to focus on formation of reactive metabolites arising from CYP450-mediated phase I reactions. This bias is based on the fact that the majority (i.e., >75%) of xenobiotic metabolism reactions are catalyzed by these enzymes, and actually only five isoforms (1A2, 2C9, 2C19, 2D6, and 3A4) account for about 90% of these reactions. This creates a significant gap in the study of reactive metabolites that may arise from biotransformation of compounds through non-CYP-mediated pathways using the traditional experimental setups. Additionally, although the major substrate classes of many of the CYP isozymes have been determined, there remains a significant number of "orphan" enzymes for which the major substrates are yet to be unraveled.[67] The possibility therefore exists that such orphan enzymes might play an important yet unappreciated role in the biotransformation of unique natural products into reactive metabolites.

■ NEW DEVELOPMENTS

Incorporation of metabolite identification modules bundled into LC-MS instrumentation software to aid greatly in in vitro experimentation data analysis is a feature that is becoming increasingly commonplace. This is in addition to the development of more powerful and efficient separation techniques such as ultra-high-performance (UHPLC) and nanoflow liquid chromatography coupled to even more sensitive mass spectrometers.[65] Such instruments have the added advantage of improving compound resolution while greatly reducing analysis run-times, thereby minimizing sample analysis turn-around times. With such equipment, it becomes feasible to carry out routine in vitro reactive metabolite screening even on large libraries of natural products in the early stages of drug discovery.

To understand the toxic effects of specific classes of reactive metabolites, a growing effort to catalogue their specific targets, mostly endogenous proteins, has been established in recent years. By building databases of known target proteins as reported in diverse literature sources arising from these metabolites, it is hoped that in the future it will be easy not only to predict the biotransformation of reactive metabolites from any xenobiotic compound but also to anticipate the resultant toxic effects in humans.[23,68]

■ USE OF INFORMATION GENERATED

Data from reactive metabolite studies in early drug discovery can be used in decision-making regarding the feasibility of proceeding to subsequent stages of the development process using the unmodified compound.[69−71] In some cases, it may be necessary to modify the chemical composition of the product, taking care not to adversely affect its pharmacological activity in the process. In the case of natural products, these endeavors may prove to be quite demanding especially due to the fact that many of their biosynthetic pathways result in compounds for which the stereochemistry is very precise and intimately coupled to their pharmacological activity. Synthetic modification of such compounds to minimize their metabolic activity must therefore be effected in a manner that does not adversely disrupt such stereochemical properties. In other instances, it may be the case that the functional components responsible for reactive metabolite formation form part of the compound's pharmacophore and therefore must be replaced or altered in a manner that does not adversely change activity.[72] Here, the application of bioisosterism would be useful. However, this must be done in an appropriate context because bioisosteres rely on biochemical mimicry rather than on physicochemical properties.[73]

An example of how these approaches have been employed is in the case of the antimalarial natural product febrifugine (**38**), derived from the Chinese herb *Chang Shan* used traditionally in the treatment of malaria but associated with hepatotoxicity

dx.doi.org/10.1021/np200786j | J. Nat. Prod. 2012, 75, 507−513

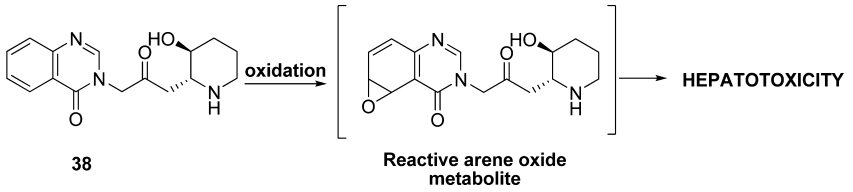


Figure 10. Biotransformation of febrifugine (**38**) to hepatotoxic reactive metabolite.

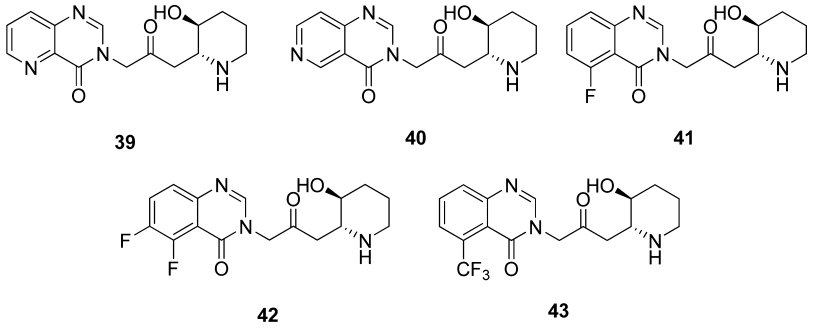


Figure 11. Synthetic analogues of febrifugine with markedly reduced hepatotoxic effects.

thought to be caused by formation of reactive metabolites (Figure 10).

Synthesis of febrifugine analogues, as shown in Figure 11, in which the formation of reactive metabolites was minimized through chemical modification of the sites of metabolism, resulted in compounds (**39**–**43**) with much lower toxicity when tested on rat hepatocytes but which retained antiplasmodial potency.[74] The introduction of electron-withdrawing or electron-deficient substituents in the aryl ring, a commonly used strategy in medicinal chemistry to block metabolism, was crucial to this success.[75]

CONCLUSIONS

Investigating the potential of natural products with promising pharmacological activity to exert toxic effects due to biotransformation into reactive metabolites is now commonly carried out routinely in early drug discovery. Developments in information technology, analytical chemistry, and toxicology have greatly contributed to research in this field in the six decades since reactive metabolites were first hypothesized and reported. Incorporation of routine reactive metabolite studies in the early phases of drug discovery provides information on likely liabilities and the opportunity to consider carrying out synthetic modifications on natural product molecules aimed at eliminating their tendency to undergo such undesirable metabolism while retaining activity. To achieve this goal, multidisciplinary efforts incorporating expertise from different fields including natural product and computational sciences, biochemistry, and analytical chemistry as well as synthetic chemistry need to be incorporated.

AUTHOR INFORMATION

Corresponding Author

*Tel: +27 21 650 2553. Fax: + 27 21 650 5195. E-mail: Kelly.Chibale@uct.ac.za.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

Financial support from the following sources is gratefully acknowledged: the South African National Research Foundation (NRF); the South African Research Chairs Initiative (SARChI) of the Department of Science and Technology (DST) administered through the NRF; the South African Medical Research Council (MRC); and the University of Cape Town.

DEDICATION

Dedicated to Dr. Gordon M. Cragg, formerly Chief, Natural Products Branch, National Cancer Institute, Frederick, Maryland, for his pioneering work on the development of natural product anticancer agents.

REFERENCES

(1) Newman, D. J.; Cragg, G. M.; Snader, K. M. *Nat. Prod. Rep.* **2000**, *17*, 215–234.
(2) Li, J. W. H.; Vederas, J. C. *Science* **2009**, *325*, 161–165.
(3) Hamilton, G. R.; Baskett, T. F. *Can. J. Anesth.* **2000**, *47*, 367–374.
(4) Guantai, E. M.; Chibale, K. *Malar. J.* **2011**, *10* (Suppl.1), S2 (15 March 2011).
(5) Wells, T. N. C. *Malar. J.* **2011**, *10* (Suppl. 1), S3 (15 March 2011).
(6) Mahajan, A.; Kumar, V.; Chibale, K. *Bioorg. Med. Chem.* **2009**, *17*, 2236–2275.
(7) Newman, D. J.; Cragg, G. M. *J. Nat. Prod.* **2007**, *70*, 461–477.
(8) Harvey, A. L. *Drug Discovery Today* **2008**, *13*, 894–901.
(9) Chin, Y.-W.; Balunas, M. J.; Chai, H.-B.; Kinghorn, A. D. *AAPS J.* **2006**, *8*, 239–253.
(10) Parkinson, A. In *Casarett & Doull's Toxicology, The Basic Science of Poisons*, 5th ed.; Klaassen, C. D., Ed.; McGraw-Hill, Inc.: New York, 1996; Chapter 6, pp 113–186.
(11) Testa, B.; Krämer, S. D. *The Biochemistry of Drug Metabolism: Principles, Redox Reactions, Hydrolyses*; VHCA: Zurich and Wiley-VCH: Weinheim, 2008; pp 25–27.
(12) Thomas, G. *Medicinal Chemistry, An Introduction*; John Wiley & Sons: Chichester, UK, 2000; pp 327–372.
(13) Williams, D. A. In *Foye's Principles of Medicinal Chemistry*, 6th ed.; Lemke, T. M.; Williams, D. A., Eds.; Lippincott Williams and Wilkins: Baltimore, MD, 2008; Chapter 10, pp 253–326.

(14) Katzung, B. G., Ed. *Basic and Clinical Pharmacology*, 10th ed.; McGraw-Hill: New York, 2006; pp 104−128.
(15) Timbrell, J. *Principles of Biochemical Toxicology*, 3rd ed.; Taylor & Francis: London, 2000; pp 65−110.
(16) Hasler, J. A.; Estabrook, R.; Murray, M.; Pikuleva, I.; Waterman, M.; Capdevila, J.; Holla, V.; Helvig, C.; Falck, J. R.; Farrell, G.; Kaminsky, L. S.; Spivack, S. D.; Boitier, E.; Beaune, P. *Mol. Aspects Med.* **1999**, *20*, 1−137.
(17) Miller, E. C.; Miller, J. A. *Cancer Res.* **1947**, *7*, 468−480.
(18) Ju, C.; Uetrecht, J. P. *Curr. Drug Metab.* **2002**, *3*, 367−377.
(19) Williams, D. P. *Toxicology* **2006**, *226*, 1−11.
(20) Guengerich, F. P. *Arch. Biochem. Biophys.* **2005**, *433*, 369−378.
(21) Guengerich, F. P. *Mutat. Res.* **2001**, *488*, 195−209.
(22) Lo, F.; Lu, J.; Ma, X. *Chem. Res. Toxicol.* **2011**, *24*, 744−751.
(23) Hanzlik, R. P.; Fang, J.; Koen, Y. M. *Chem.-Biol. Interact.* **2009**, *179*, 38−44.
(24) van Bladeren, P. J. *Chem.-Biol. Interact.* **2000**, *129*, 61−76.
(25) Park, B. K.; Naisbitt, D. J.; Gordon, S. F.; Ketteringham, N. R.; Pirmohamed, M. *Toxicology* **2001**, *158*, 11−23.
(26) Knowles, S. R.; Uetrecht, J.; Shear, N. H. *Lancet* **2000**, *356*, 1587−1591.
(27) Park, B. K.; Williams, D. P.; Naisbitt, D. J.; Kitteringham, N. R.; Pirmohamed, M. *Toxicol. Appl. Pharmacol.* **2005**, *207*, S425−S434.
(28) Uetrecht, J. *Annu. Rev. Pharmacol. Toxicol.* **2007**, *47*, 513−539.
(29) Shu, Y.; Johnson, B. M.; Yang, T. J. *AAPS J.* **2008**, *10*, 178−192.
(30) Takakusa, H.; Masumoto, H.; Yukinaga, H.; Makino, C.; Nakayama, S.; Okazaki, O.; Sudo, K. *Drug Metab. Dispos.* **2008**, *36*, 1770−1779.
(31) Tafazoli, S.; O'Brien, P. J. *Toxicology* **2009**, *256*, 101−109.
(32) Boelsterli, U. A. *Toxicol. Appl. Pharmacol.* **2003**, *192*, 307−322.
(33) Wolkenstein, P.; Tan, C.; Lecoeur, S.; Wechsler, J.; Garcia-Martin, N.; Charue, D.; Bagot, M.; Beaune, P. *Chem.-Biol. Interact.* **1998**, *113*, 39−50.
(34) Rodriguez, R. J.; Acosta, D. Jr. *Toxicology* **1997**, *117*, 123−131.
(35) Neuman, M. G.; Shear, N. H.; Jacobson-Brown, P. M.; Katz, G. G.; Neilson, H. K.; Malkiewicz, I. M.; Cameron, R. G.; Abbott, F. *Clin. Biochem.* **2001**, *34*, 211−218.
(36) Dietz, B. M.; Bolton, J. L. *Chem.-Biol. Interact.* **2011**, *192*, 72−80.
(37) Yan, Z.; Zhong, H. M.; Maher, N.; Torres, R.; Leo, G. C.; Caldwell, G. W.; Huebert, N. *Drug Metab. Dispos.* **2005**, *33*, 1867−1876.
(38) Falzon, M.; McMahon, J. B.; Schuller, H. M.; Boyd, M. R. *Cancer Res.* **1986**, *46*, 3484−3489.
(39) Druckova, A.; Mernaugh, R. L.; Ham, A-J. L.; Marnett, L. J. *Chem. Res. Toxicol.* **2007**, *20*, 1393−1408.
(40) Nelson, S. D. *Curr. Ther. Res. Clin. Exp.* **2001**, *62*, 885−899.
(41) Li, A. P. *Chem.-Biol. Interact.* **2002**, *142*, 7−23.
(42) Fontana, E.; Dansette, P. M.; Poli, S. M. *Curr. Drug Metab.* **2005**, *6*, 413−454.
(43) Egan, W. J.; Zlorkanik, G.; Grootenhuis, P. D. J. *Drug Discovery Today* **2004**, *4*, 381−387.
(44) Kumar, N.; Hendriks, B. S.; Janes, K. A.; de Graaf, D.; Lauffenburger, D. A. *Drug Discovery Today* **2006**, *11*, 806−811.
(45) Vendani, A.; Dobler, M.; Lill, M. A. *Toxicol. Appl. Pharmacol.* **2005**, *207*, S398−S407.
(46) de Groot, M. J. *Drug Discovery Today* **2006**, *11*, 601−606.
(47) Chen, Y.; Monshouwer, M.; Fitch, W. L. *Pharm. Res.* **2007**, *24*, 248−257.
(48) Crivori, P.; Poggesi, I. *Eur. J. Med. Chem.* **2006**, *41*, 795−808.
(49) Zheng, M.; Luo, X.; Shen, Q.; Wang, Y.; Du, Y.; Zhu, W.; Jiang, H. *Bioinformatics* **2009**, *25*, 1251−1258.
(50) Sun, H.; Scott, D. O. *Chem. Biol. Drug Des.* **2010**, *75*, 3−17.
(51) Valerio, L. G. Jr. *Toxicol. Appl. Pharmacol.* **2009**, *241*, 356−370.
(52) Ekins, S.; Andreyev, S.; Ryabov, A.; Kirillov, E.; Rakhmatulin, E. A.; Bugrim, A.; Nikolskaya, T. *Exp. Opin. Drug Metab. Toxicol.* **2005**, *1*, 303−324.
(53) Zhang, D.; Zhu, M.; Humphreys, W. G., Eds. *Drug Metabolism in Drug Design and Development: Basic Concepts and Practice*; John Wiley & Sons: New York, 2008; pp 353−358.
(54) Haripasad, N.; Sane, R. S.; Strom, S. C.; Desai, P. B. *Toxicol. in Vitro* **2006**, *20*, 135−153.
(55) Damsten, M. C.; van Vugt-Lussenburg, B. M. A.; Zeldenthuis, T.; de Vlieger, J. S. B.; Commandeur, J. N. M.; Vermeulen, N. P. E. *Chem.-Biol. Interact.* **2008**, *171*, 96−107.
(56) Chen, Y.; Monshouwer, M.; Fitch, W. L. *Pharm. Res.* **2007**, *24*, 248−257.
(57) Evans, D. C.; Watt, A. P.; Nicoll-Griffith, D. A.; Baillie, T. A. *Chem. Res. Toxicol.* **2004**, *17*, 3−16.
(58) LeBlanc, A.; Shiao, T. C.; Roy, R.; Sleno, L. *Rapid Commun. Mass Spectrom.* **2010**, *24*, 1241−1250.
(59) Laine, J. E.; Auriola, S.; Pasanen, M.; Juvonen, R. O. *Toxicol. in Vitro* **2011**, *25*, 411−425.
(60) Zhou, S.; Chan, E.; Lim, L. Y.; Boelsterli, U. A.; Li, S. C.; Wang, J.; Zhang, Q.; Huang, M.; Xu, A. *Curr. Drug Metab.* **2004**, *5*, 415−442.
(61) Zhou, S.; Chan, S. Y.; Goh, B. C.; Chan, E.; Duan, W.; Huang, M.; McLeod, H. L. *Clin. Pharmacokinet.* **2005**, *44*, 279−304.
(62) Madsen, K. G.; Olsen, J.; Skonberg, C.; Hansen, S. H.; Jurva, U. *Chem. Res. Toxicol.* **2007**, *20*, 821−831.
(63) Johansson, T.; Weidolf, L.; Jurva, U. *Rapid Commun. Mass Spectrom.* **2007**, *21*, 2323−2331.
(64) Tolonen, A.; Turpeinen, M.; Pelkonen, O. *Drug Discovery Today* **2009**, *14*, 120−133.
(65) Ma, S.; Zhu, M. *Chem.-Biol. Interact.* **2009**, *179*, 25−37.
(66) Nassar, A-E. F.; Kamel, A. M.; Clarimont, C. *Drug Discovery Today* **2004**, *9*, 1055−1064.
(67) Guengerich, F. P.; Tang, Z.; Cheng, Q.; Salamanca-Pinzón, S. G. *Biochim. Biophys. Acta* **2011**, *1814*, 139−145.
(68) Hanzlik, R. P.; Koen, Y. M.; Theertham, B.; Dong, Y.; Fang, J. *Bioinformatics* **2008**, *8*, 95−100.
(69) Thompson, R. A.; Isin, E. M.; Li, Y.; Weaver, R.; Weidolf, L.; Wilson, I.; Claesson, A.; Page, K.; Dolgos, H.; Kenna, J. G. *Chem.-Biol. Interact.* **2011**, *192*, 65−71.
(70) Kalgutkar, A. S. *Chem.-Biol. Interact.* **2011**, *192*, 46−55.
(71) Humphreys, W. G. *Chem.-Biol. Interact.* **2011**, *192*, 56−59.
(72) Park, B. K.; Boobis, A.; Clarke, S.; Goldring, C. E. P.; Jones, D.; Kenna, J. G.; Lambert, C.; Laverty, H. G.; Naisbitt, D. J.; Nelson, S.; Nicoll-Griffith, D. A.; Obach, R. S.; Routledge, P.; Smith, D. A.; Tweedie, D. J.; Vermeulen, N.; Williams, D. P.; Wilson, I. D.; Baillie, T. A. *Nat. Rev. Drug Discovery* **2011**, *10*, 292−306.
(73) Meanwell, N. A. *J. Med. Chem.* **2011**, *54*, 2529−2591.
(74) Zhu, S.; Meng, L.; Zhang, Q.; Wei, L. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 1854−1858.
(75) Kerns, E. H.; Di, L. *Drug-like Properties: Concepts, Structure Design and Methods: from ADME to Toxicity Optimization*; Academic Press: Burlington, MA, 2008; pp 146−156.