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A new structural alert for benzimidazoles: 2,6-Dimethylphenyl substituents increase mutagenic potential and time-dependent CYP3A4 inhibition risk

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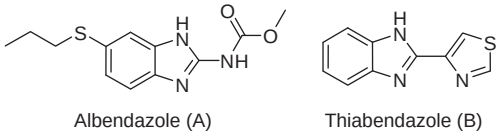
ABSTRACT

A series of 2-[(2,6)-dimethylphenyl]benzimidazole analogs displayed strong potential for mutagenicity following metabolic activation in either TA98 or TA100 *Salmonella typhimurium* strains. The number of revertants was significantly reduced by replacing the 2,6-dimethylphenyl group with a 2,6-dichlorophenyl moiety. Time-dependent CYP3A4 inhibition was also observed with a compound containing a 2-[(2,6)-dimethylphenyl] benzimidazole ring, implying risk for this scaffold to generate reactive metabolites.

The benzimidazole ring is often evaluated as a template for small-molecule drug discovery,[1] owing to its incorporation into several pharmaceutical agents (i.e., dabigatran[2] and lansoprazole[3]) and the development of powerful synthetic methods for benzimidazole assembly and functionalization.[4] In addition to its pharmacological properties, however, several liabilities have been attributed to this heterocycle;[5] the mutagenicity of albendazole (**A**), for instance, is linked to the the 2-amino benzimidazole moiety, while the pesticide thiabendazole (**B**) is also linked to increased mutagenic risk (Fig. 1).[6]

The two imidazole nitrogen atoms are often implicated in the undesirable properties of benzimidazole-containing drug candidates, as these can potentially serve as ligands for metal-containing enzymes, including cytochrome P450.[7] Indeed, single atom replacement of one of these nitrogen atoms[8] or electronic tuning of the benzimidazole ring[9] has proven to be a successful strategy to mitigate benzimidazole-associated liabilities. However, the embedded *ortho* arrangement of the two benzimidazole nitrogen atoms has drawn less concern in spite of the well-known risk of reactive intermediates derived from *ortho*- and *para*-quinone species.[10] Here we report a remarkable substituent effect on the mutagenic risk of 2-phenyl substituted benzimidazoles, whereby the latent *ortho*-iminoquinone reactivity of the benzimidazole ring is potentially unmasked by metabolism. Our data suggest that metabolic activation of remote substituents should be carefully considered when anticipating liabilities of benzimidazole-containing therapeutic agents.

In vitro profiling of benzimidazole **1** in the well-established Ames assay[11] identified an increased risk for mutagenicity as measured by the 2.06-fold increase in revertants in histidine-depleted Salmonella strain TA100 (100 μg of **1** per well). Recognizing the potential for amide hydrolysis of **1** to release a heteroaromatic amine, we profiled the 2-aminoquinoline fragment which in our hands showed no genotoxic effects.[12] Our suspicion of the 2-aminoquinoline fragment was further reduced when the 2,6-dichlorophenyl analog **2** was negative in the Ames assay (no significant increase in revertants in TA98 or TA100 strains, +/− S9 activation).[13] As the increase in revertants for **1** was observed only with S9 metabolic activation, we wondered whether oxidative metabolites were responsible for the increased mutagenic risk. An in silico metabo-



Albendazole (A) Thiabendazole (B)

Figure 1. Pharmacologically-active benzimidazoles with known genotoxic risk.

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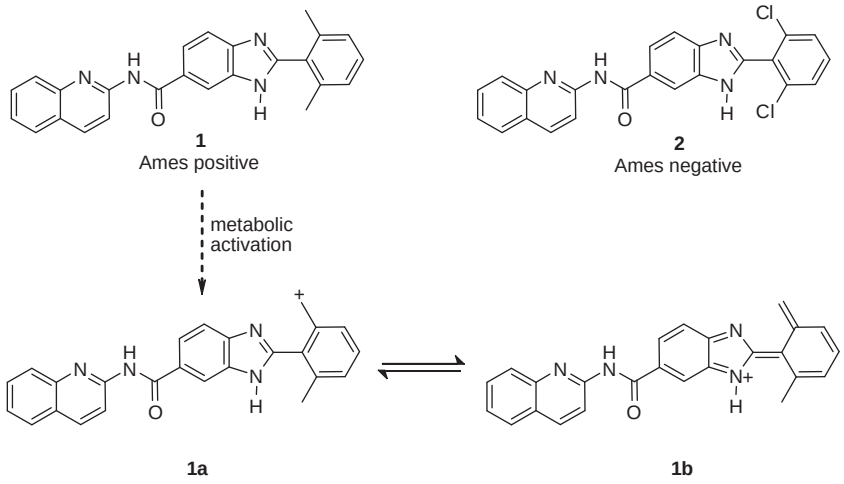


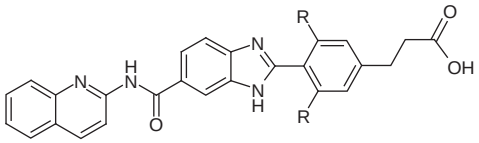
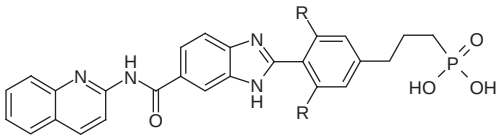
Figure 2. Metabolic activation of 2-[(2,6)-dimethylphenyl]benzimidazole and resonance-stabilization to iminoquinone **1b**.

lism model predicted the methyl groups on **1** to be likely sites of oxidation.[14] Upon recognition that an intermediate of this oxidation process (**1a**) might equilibrate through resonance-stabilization with the *ortho*-iminoquinone species **1b**, we began to suspect the seemingly innocuous 2,6-dimethylphenyl group as the potential culprit (Fig. 2).

Indeed, the mutagenicity of 2-[(2,6)-dimethylphenyl)benzimidazoles proved to be quite consistent, as compounds **3a** and **4a** bearing a variety of peripheral substitution patterns tested positive in the Ames assay (Table 1). In each case, replacement of the methyl groups with chlorine atoms dramatically reduced the genotoxic risk, as dichloro analogs **3b** and **4b** showed no significant increase in reverting mutations in the Ames assay in the presence and absence of S9 metabolic activation (Table 1).

The minimal toxicophore for the molecules described herein is likely the 2-[(2,6)-dimethylphenyl]benzimidazole core, as this compound itself (**5**) at 50 μg/well led to a 2.58-fold increase in revertants following S9 metabolic activation (Fig. 3). Compound **6**

Table 1
Mutagenic risk for benzimidazole analogs **3–4**

Compound	R	Maximum revertant increase −S9 activation (dose, strain)	Maximum revertant increase +S9 activation (dose, strain)
	**3a**: R = Me **3b**: R = Cl	5.86 (100 μg, TA98) NS[a]	14.57 (300 μg, TA98) NS
	**4a**: R = Me **4b**: R = Cl	2.48 (1000 μg, TA100) NS	2 (1000 μg, TA100) NS

[a] NS = non-significant increase in revertants.

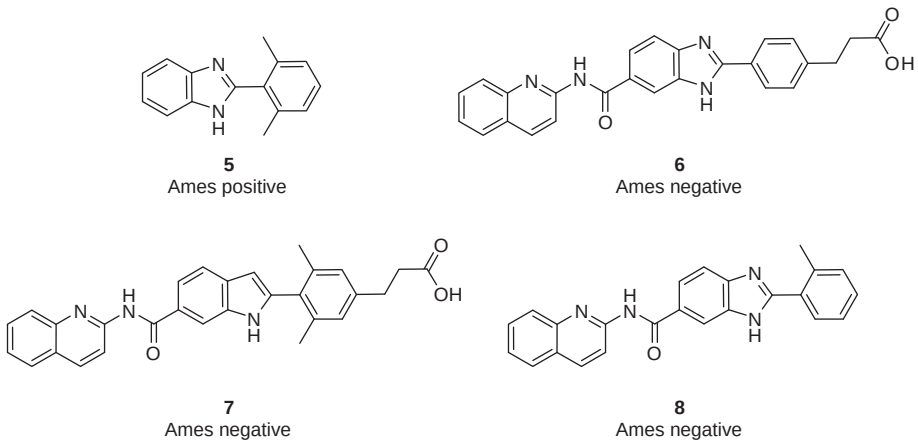


Figure 3. Ames results for compounds **5**–**8**.

which lacks the two methyl groups in **3a** was negative in the Ames assay (no increase in revertants). Moreover, when one of the nitrogen atoms of **3a** was replaced with a carbon to preclude formation of any *ortho*-iminoquinone species, the corresponding indole **7** also led to no increase in revertants with and without metabolic activation. Interestingly, deletion of one methyl group afforded the Ames-negative analog **8** (no increase in revertants +/− S9 activation). We speculate the di-*ortho*-substitution in **1** induces a twist around the biaryl bond that renders the benzimidazole nitrogens more accessible to bind the CYP3A4 iron center and facilitate subsequent oxidation, whereas the monomethyl analog **8** undergoes lesser metabolic activation due to steric shielding of the benzimidazole nitrogen atoms. Overall, for 3 of the 4 Ames-positive analogs we identified in this series, a greater number of revertants were observed in the presence of S9 metabolic activation.

To investigate whether our strategy to reduce metabolism within the benzimidazole framework could be extended beyond mitigating genotoxicity risk, we measured the inhibitory effect of **1** and **8** on CYP3A4-mediated biotransformation, including irreversible (or time-dependent) inactivation.[15] In the absence of compound pre-incubation, **1** was a moderate inhibitor of CYP3A4 (IC_{50} = 7.3 μM), and the monomethyl analog **8** was a much weaker CYP3A4 inhibitor (IC_{50} >50 μM). This result is consistent with our Ames findings for **1** and **8**, where we proposed stronger binding of **1** to CYP3A4 increased the potential for metabolic activation and subsequent genotoxic risk. Pre-incubation (30 min) of **1** with human liver microsomes and the cofactor NADPH further exacerbated this CYP3A4 inhibition as evidenced by the 2.7-fold shift in IC_{50} (IC_{50} following pre-incubation = 2.7 μM), whereas compound **8** still showed no CYP3A4 inhibition under these conditions (IC_{50} >50 μM). The observation of time-dependent CYP3A4 inhibition with **1** implies formation of a reactive metabolite which, given the lack of irreversible inhibition with **8**, is most likely associated with the 2,6-dimethylphenyl ring. The congruence of two unrelated in vitro profiling assays (Ames and time-dependent CYP3A4 inhibition) which both identify risk for 2-[(2,6)-dimethylphenyl]benzimidazoles argues for careful consideration of this structural motif in drug candidate discovery.

In summary, we have identified a remarkable impact of 2,6-dimethylphenyl substitution patterns on the overall mutagenic risk of 2-substituted benzimidazoles. The latent reactivity of these fragments was further linked to time-dependent CYP3A4 inhibition and could be attenuated by removing one of the metabolic triggers. This work suggests additional structural alerts for the benzimidazole ring that reasonably anticipate remote stabilization of potentially-reactive *ortho*-iminoquinone species should be considered.

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13. Representative synthetic procedures for all compounds may be found in WO2008048991. Analytical data for compound **1**: HR-MS: calcd 393.1715, found 393.1729; ^{1}H NMR (DMSO-d_6): δ 11.36 (s, broad, 1H), 8.54 (s, 1H), 8.46 (d, J = 9.09 Hz, 1H), 8.38 (d, J = 9.09 Hz, 1H), 8.17 (d, J = 8.59 Hz, 1H), 7.99 (d, J = 7.33 Hz, 1H), 7.93 (d, J = 8.08 Hz, 1H), 7.86 (d, J = 8.59 Hz, 1H), 7.77 (t, 1H), 7.56 (t, 1H), 7.46 (t, 1H), 7.30 (d, J = 7.58 Hz, 1H), 2.19 (s, 6H). Compound **2**: HR-MS: calcd 433.0636, found 433.0625; ^{1}H NMR (MeOD): δ 8.49 (s, broad, 0.5 H), 8.40 (q, 2H), 8.36 (s, broad, 0.5H), 8.06 (d, broad, J = 8.59 Hz, 1H), 7.93–7.88 (m, 2H), 7.72 (t, 2H), 7.63–7.55 (m, 3H), 7.52 (t, 1H).
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