p-Azidosalicyl-5-amino-6-phenoxybenzimidazole 
Photolabels the N-terminal 63-103 Amino Acids of 
Haemonchus contortus β-Tubulin 1*

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Benzimidazoles (BZ) are broad spectrum anthelmints thought to exert their effects by interacting with and disrupting the functions of microtubules. However, direct biochemical evidence for binding between BZ and tubulin has not been shown nor is it known what sequences in tubulin interact with BZ. In this study, a photoactive analogue of 2-acetamido-5-(3-aminophenoxy) benzimidazole that has biological activity similar to other benzimidazoles was synthesized and used to photolabel cell lysates from the parasitic nematode of sheep Haemonchus contortus. The photoactive analogue, 2-acetamido-5-[3-(4-azido-3,125I-salicylamido)phenoxy]benzimidazole or 125I-ASA-BZ, was shown to photolabel a 54-kDa protein that was specifically immunoprecipitated with anti-tubulin monoclonal antibodies. Tubulin photoaffinity labeling by 125I-ASA-BZ was also inhibited with molar excess of various BZ analogues and colchicine. Interestingly, 125I-ASA-BZ photoaffinity-labeled the β- and not the α-subunits of tubulin. Proteolytic digestion of 125I-ASA-BZ-labeled tubulin with Staphylococcus aureus V8 proteinase revealed one major peptide with an apparent molecular mass of 3.5 kDa. Exhaustive digestion of 125I-ASA-BZ-labeled β-tubulin with trypsin resulted in two fractions containing radioactive peptides. Protein sequencing of the high performance liquid chromatography-purified tryptic ASA-BZ-photolabeled peptides identified the N-terminal 63-77 and 78-103 sequences as the BZ binding domain.

The frequent application of BZ in the control of parasitic nematodes has led to the rapid selection of BZ-resistant populations (16, 17). Recently, mutations in tubulin genes have been correlated with the development of BZ resistance (18–20), and mutations conferring BZ resistance have been mapped to the locus encoding the β-tubulin (21–27). However, without direct biochemical evidence for binding between tubulin and BZ, it is not clear if the mutations in the β-tubulin genes that confer resistance to BZ affect direct BZ binding to β-tubulin. In addition, we have shown recently (5) that BZ are substrates for the P-glycoprotein drug efflux pump that mediates the multidrug resistance phenotype in tumor cells (28, 29) and in some parasites (30, 31). Therefore, an enhanced drug efflux mechanism in resistant H. contortus could confer resistance to BZ.

To further characterize the biochemical and molecular basis for the action of BZ, it was of interest to show directly the receptor(s) of BZ using a photoaffinity labeling assay. This approach has been used to identify the receptor and the ligand binding domain on such a receptor for many ligands including several antimitic drugs. Photolabile analogues of vinblastine, colchicine, rhizoxin, and taxol have been used previously to demonstrate their direct binding to tubulin and to identify their photolabeled sequences (32–35). Moreover, in support of the latter approach, a recent study (36) using high resolution images of taxol-bound tubulin has provided structural evidence for taxol binding domain that is consistent with earlier results using a photoactive analogue of taxol (35). In this report, we describe the synthesis of the photoactive-radioactive analogue of 2-acetamido-5-(3-aminophenoxy) benzimidazole, 125I-ASA-BZ, and its use in a photoaffinity labeling assay. The results of this study show for the first time the direct binding of BZ to...
tubulin. In addition, we show that the $^{125}$I-ASA-BZ binding domain is localized to a 36-amino acid sequence (Ala$^{63}$-Lys$^{103}$) in the N-terminal of $\beta$-tubulin. The implication of these findings with respect to tubulin drug binding sites and BZ species selectivity will be discussed.

**EXPERIMENTAL PROCEDURES**

**Materials—**2-Acetamido-5-(3-aminophenoxy)benzimidazole (amino-BZ) (a fenbendazole analogue) (Fig. 1, top) was a gift from Hoechst, Germany, and N-hydroxyxysuximidyl-4-azidosalicylic acid (NHS-ASA) was purchased from Pierce. Pure samples of nonlabeled mebendazole, oxfendazole, and thiaobendazole were synthesized in the laboratory of J. Missiaena, ON, Syntex Inc. Palo Alto, CA, and Merck Frosst, Kirkland, QC, respectively. Colchicine was purchased from Sigma. Sodium $^{125}$Iiodide and monoclonal antibodies to chicken brain tubulin were obtained from Amersham. Sequence grade proteases from the mucosa of the abomasum and washed extensively with 20% dimethylsulfoxide to give a final concentration of 50 nm. For inhibition of photoaffinity labeling, protein fractions were preincubated with a molar excess of cold BZ analogues before the addition of $^{125}$I-ASA-BZ. At the end of the incubation period (30–60 min), samples were placed on ice for 10 min followed by a 10-min UV irradiation at 254 nm (UV Stratalinker 1800, Stratagene).

**Protease Cleavage of $^{125}$I-ASA-BZ—**The photoaffinity-labeled protein which corresponded to tubulin was excised and digested with Staphylococcus aureus V8 protease (10–20 $\mu$g/ml slice) in the well of a 15% Laemmli gel as originally described by Cleveland et al. (46). For trypsin digestion, 2 mg of recombinant p12–16 tubulin was photoaffinity-labeled with 50 nm $^{125}$I-ASA-BZ. Photoaffinity-labeled tubulin was reduced with dithiothreitol and alkylated with iodoacetamide before the addition of trypsin. The digestion was carried out in 0.05 M (NH$_4$)$_2$SO$_4$, pH 8.0, for 24 h at a trypsin to protein ratio of 1:25 (w/w). The resultant tryptic digest was dried by speed vacuum and prepared for hplc analysis.

**Results**

**Photoaffinity Labeling of Tubulin with $^{125}$I-ASA-BZ—**To determine the BZ receptor(s) in parasitic nematodes, we have synthesized a photoactive analogue of BZ (Fig. 1) and used it in a photoaffinity labeling assay. The sheep nematode H. contortus was used as a source of tubulin. Fig. 2A, lane 1, shows a Coomassie Blue staining of the cytosolic proteins from adult worms of H. contortus resolved on SDS-PAGE. Photoaffinity labeling was performed by incubating 100 $\mu$g of cytosolic proteins with 50 nm $^{125}$I-ASA-BZ followed by UV-irradiation and SDS-PAGE (see "Experimental Procedures"). The results in Fig. 2B show a single protein with a molecular mass of approximately 54 kDa that was specifically photolabeled with $^{125}$I-ASA-BZ (lane 2). The incubation of cytosolic proteins with $^{125}$I-ASA-BZ, but without UV-irradiation, did not result in the photolabeling of the 54-kDa protein (Fig. 2B, lane 1). The latter
Increasing concentrations (10–200 nM) of 125I-ASA-BZ were incubated with 125I-ASA-BZ but not exposed to UV light. Contortus results demonstrate that the 54-kDa protein in H. contortus lanes 1 and 2, respectively. The positions of molecular mass markers are shown on the gel slice was determined by photoaffinity labeling of 54-kDa protein in the absence (lane 1, 5, 10, and 50 μM amino-BZ (lanes 3, 4, 5, and 6, respectively). The positions of molecular mass markers are shown on the left of lane 1. A, C shows photoaffinity labeling of H. contortus fractions with increasing concentrations (10–200 nM) of 125I-ASA-BZ (inset). The 54-kDa photoaffinity-labeled protein was excised, and the radioactivity in each gel slice was determined by γ counting. A plot of the ASA-BZ incorporation in the 54-kDa protein versus the concentration of 125I-ASA-BZ is shown in C.

Results demonstrate that the 54-kDa protein in H. contortus cytosolic fraction is covalently cross-linked by ASA-BZ. To confirm the specificity of 125I-ASA-BZ binding to the 54-kDa protein, cytosolic proteins were incubated with 50 nm 125I-ASA-BZ in the presence of increasing molar concentrations of the unmodified amino-BZ. The results in lanes 2–6 of Fig. 2B show the photoaffinity labeling of 54-kDa protein in the absence (lane 2) and in the presence of 1–50 μM concentration of the amino-BZ (lanes 3–6). The photoaffinity labeling of the 54-kDa protein appears to be specific since the presence of excess unmodified amino-BZ inhibited its photolabeling with 125I-ASA-BZ in a dose-dependent manner. Furthermore, the addition of higher concentrations of 125I-ASA-BZ (50–200 nM) to cytosolic extracts (100 μg) showed saturable photoaffinity labeling of a 54-kDa protein (Fig. 2C). Taken together, these results show that the photolabeling of the 54-kDa protein with 125I-ASA-BZ in cytosolic extracts from H. contortus is specific and saturable.

To confirm the identity of the 54-kDa ASA-BZ-photolabeled protein, 125I-ASA-BZ-photolabeled cytosolic fractions were incubated with anti-tubulin monoclonal antibodies (mAbs), and the immunoprecipitated proteins were resolved on SDS-PAGE. The results in Fig. 3 show a 54-kDa protein immunoprecipitated from H. contortus with anti-tubulin mAbs (lane 3), but not with an irrelevant IgG2a (lane 1). Similar results were also obtained when a cell extract from E. coli, which overexpresses the H. contortus β12-16 tubulin gene, was photoaffinity-labeled with 125I-ASA-BZ and immunoprecipitated with anti-tubulin mAbs or an irrelevant IgG2a (Fig. 3, lane 4 or 2, respectively). These results confirm the identity of the 54-kDa protein as tubulin and provide the first direct evidence for BZ binding to tubulin.

The above results in Fig. 3 (lane 4) suggest that β-tubulin expressed in E. coli is photoaffinity-labeled with ASA-BZ; hence, BZ can interact with recombinant β-tubulin monomer(s). However, it is unclear whether α-tubulin is also photoaffinity-labeled with 125I-ASA-BZ. To determine if one or both tubulin subunits are photoaffinity-labeled with ASA-BZ, cytosolic extracts from H. contortus were photoaffinity-labeled with 125I-ASA-BZ, separated on gradient SDS-PAGE (4 to 14%), and transferred to nitrocellulose membrane for Western blot analysis with anti-α- and/or β-tubulin mAbs. The results in lane 3 of Fig. 4 show a single polypeptide detected with anti-β-tubulin mAb. Lane 4 of Fig. 4 shows the two subunits of tubulin when the sample identical with lane 3 is probed with both anti-α- and β-tubulin mAbs. These results demonstrate that the photoaffinity-labeled protein (Fig. 4, lanes 1 and 2) co-migrates with β-tubulin subunit (lane 3). In addition, the photoaffinity-labeled subunit in H. contortus (lane 1) co-migrates with the recombinant β-tubulin from E. coli extracts. Similar results were also obtained when purified mammalian brain tubulin was photoaffinity-labeled and fractionated on gradient SDS-PAGE to separate α- and β-tubulin subunits (data not shown) except that the mammalian α-migrates slower than the β-tubulin as reported previously (43).

Effect of BZ Analogues and Colchicine on the Binding of 125I-ASA-BZ to Tubulin—It was previously demonstrated that...
the binding affinity of different analogues of BZ to mammalian tubulin correlated with their ability to inhibit tubulin polymerization (9) or inhibit \(^{125}\)I-BZ binding to H. contortus crude extracts (49). Given the binding specificity of \(^{125}\)I-ASA-BZ to tubulin in H. contortus and to confirm the specificity of \(^{125}\)I-ASA-BZ photoaffinity labeling to tubulin drug binding site(s), it was of interest to determine whether different BZ analogues interacted with the same or different binding domain(s) as the \(^{125}\)I-ASA-BZ. The results in Fig. 5 show \(^{125}\)I-ASA-BZ photoaffinity labeling of cytosolic extracts from H. contortus in the absence (lane 1) and in the presence of a 1000-fold molar excess of amino-BZ, mebendazole, oxendazole, colchicine, or thiabendazole (lanes 2–6, respectively). Mebendazole, a potent BZ analogue, was very effective in inhibiting the photoaffinity labeling of tubulin with \(^{125}\)I-ASA-BZ (lane 3). Thiabendazole, a BZ analogue that is structurally distinct from BZ carbamates, was virtually without effect (lane 6). Oxendazole, a sulfoxide metabolite of fenbendazole, was less inhibitory to the photoaffinity labeling of tubulin by \(^{125}\)I-ASA-BZ (Fig. 5; lanes 4 versus 3). Colchicine, previously shown to interact at or near BZ binding site(s) in mammalian tubulin (9), was not a good inhibitor of ASA-BZ photoaffinity labeling of tubulin (lane 5). Taken together, these results suggest that the binding of ASA-BZ to tubulin occurs at a physiologically relevant BZ binding domain(s).

The Monomer \(\beta\)-Tubulin in E. coli Encodes a Similar BZ Binding Domain as Native Tubulin Dimer from H. contortus—The results in Fig. 3 (lane 4) show that \(\beta\)-12–16 tubulin monomer from cell extracts of E. coli is photoaffinity-labeled by \(^{125}\)I-ASA-BZ. However, it was not clear if the binding specificity of the recombinant \(\beta\)-12–16 tubulin monomer is similar to native tubulin from H. contortus. The results in Fig. 6 show the photoaffinity labeling of \(\beta\)-12–16 tubulin in the absence (lane 1) and in the presence of amino-BZ, colchicine, or thiabendazole (lanes 2–4, respectively). The presence of molar excess of amino-BZ completely inhibited the photoaffinity labeling of \(\beta\)-12–16 tubulin by \(^{125}\)I-ASA-BZ (lane 2), while the presence of colchicine or thiabendazole showed less inhibition (lanes 3 and 4, respectively).
ences in the number or the electrophoretic mobility of the photolabeled peptides (data not shown). Taken together, these results suggest that $^{125}$I-ASA-BZ binding to recombinant and native tubulin is similar. In addition, the identity of the 54-kDa photoaffinity-labeled protein as $\beta$-tubulin in $H.\ contortus$ is further confirmed since identical peptide maps were obtained when recombinant $\beta_{12–16}$ tubulin and the 54-kDa (unresolved $\alpha$- and $\beta$-tubulin) photoaffinity-labeled proteins were digested with V8 proteinase.

$^{125}$I-ASA-BZ-photolabeled Amino Acid Sequences of $\beta$-Tubulin—Given that $^{125}$I-ASA-BZ photolabels similar peptides in native and recombinant $\beta_{12–16}$ tubulin and the low amounts of tubulin in $H.\ contortus$ (40) which render the purification of $^{125}$I-ASA-BZ-photolabeled peptides very difficult, we used recombinant tubulin to identify the BZ-photolabeled domain. The results in Fig. 8 show the separation of $\beta$-tubulin tryptic peptides by hplc using reverse-phase chromatography. Analysis of the above fractions for radioactivity indicated the presence of two radioactive peaks in fractions 45 and 54, respectively (Fig. 8A). Both fractions were collected and further purified using a shallower gradient of acetonitrile. Fig. 8B shows hplc tracing and radioactivity profile for $^{125}$I-ASA-BZ-photolabeled peptides in fractions 45 and 54 following several runs of purification (panels a and b or c and d, respectively). The amino acid sequence of the resultant tryptic peptides was determined by Edman degradation (see "Experimental Procedures"). The sequence of the tryptic peptide in fraction 45 was shown to encode the following amino acids H$_2$N-Ala-Val-Leu-Val-Asp-Leu-Glu-Pro-Gly-Thr-Met-Asp-Ser-Val-Arg-COOH. The latter sequence corresponded to residues 63–77 of $H.\ contortus$ $\beta_{12–16}$ tubulin. Fraction 54 was shown to contain a larger tryptic peptide of 26 residues long (78–103; H$_2$N-Ser-Gly-Pro-Phe-Gly-Ala-Leu-Phe-Arg-Pro-Asp-Asp-Phe-Val-Phe-Gly-Gln-Ser-Gly-Ala-Gly-Asn-Trp-Ala-Lys-COOH). Interestingly, the amino acid sequence of the second tryptic peptide mapped immediately following the N-terminal of the first tryptic peptide. In an effort to determine the amino acids that are specifically modified by $^{125}$I-ASA-BZ, samples were removed following each cycle of the Edman degradation, and their radioactivity was quantitated. A significant amount of radioactivity was found to be associated with the third and the fourth cycle of the Edman degradation of peptides 45 and 54, respectively. Thus, the amino acids Leu$^{65}$ and Phe$^{80}$ represent the modification sites by ASA-BZ in peptides 45 and 54, respectively.

**DISCUSSION**

In this study we show that a photoactive analogue of BZ interacts directly and specifically with tubulin from $H.\ contortus$. The specificity of $^{125}$I-ASA-BZ toward tubulin was confirmed by the inhibition of photoaffinity labeling in the presence of BZ analogues. These results are consistent with earlier predictions that the high affinity BZ binding to nematode homogenate is due to tubulin (6–8, 14, 50). Furthermore, the observations that thiabendazole, which lacks the methyl carbamate and the sulfoxide BZ analogue, oxendazole, competes poorly for BZ binding to tubulin agree with previous drug binding studies (6–8, 49). Colchicine, which is structurally unrelated to BZ, was less inhibitory to the photolabeling of
tubulin with $^{125}$I-ASA-BZ. An earlier study (6) has shown that colchicine reduces the binding of BZ to both mammalian tubulin and nematode homogenate. Moreover, BZ binding to fungal extracts was competitively inhibited by colchicine and trichodizole and not by other unrelated anti-microtubule agents (51). However, binding of colchicine to tubulin is characteristically different from that of BZ compounds, and the selectivity of the latter for lower eukaryotic protein has not been demonstrated different from that of BZ compounds, and the selectivity of the latter for lower eukaryotic protein has not been demonstrated.

The development of BZ resistance in nematodes and other BZ-sensitive organisms has often been associated with changes in the genes encoding $\beta$-tubulin. Our data provide direct evidence that indeed $\beta$-tubulin is the acceptor protein for BZ in parasitic nematodes. This conclusion is further bolstered by the observation, in this study, that recombinant $\beta 12-16$ tubulin monomers are specifically photoaffinity-labeled by ASA-BZ. Thus, $\beta$-tubulin alone appears to bind BZ while the $\alpha$-subunits are less essential for BZ binding to microtubule. This finding is not exclusive to BZ, as photoactive analogues of several anti-mitotic drugs (taxol, colchicine, and rhizoxin) that bind to and inhibit the functions of microtubule have been shown to bind $\beta$- and not $\alpha$-tubulin (33-35). This is in contrast with other anti-tubulin drugs (e.g. vinblastine) that photoaffinity label both $\alpha$- and $\beta$-tubulin (32). The significance of the observed difference in antimitotic drug interactions with tubulin subunits is currently not clear.

The cleavage of $^{125}$I-ASA-BZ-photolabeled $\beta$-tubulin with trypsin yielded two labeled peptides. N-terminal sequencing of $^{125}$I-ASA-BZ-photolabeled tryptic peptides has localized the BZ binding domain to a span of 36 amino acids (Ala$^{63}$ to Lys$^{103}$) in $\beta$-tubulin. The assignment ASA-BZ binding to this region of $\beta$-tubulin is consistent with our V8 protease mapping results which showed one major $^{125}$I-ASA-BZ photolabeled peptide of $\sim 3.5$ kDa on SDS-PAGE. Analysis of $\beta$-tubulin amino acid sequence for all possible V8 cleavage sites revealed a 34-amino acid peptide (Ser$^{75}$ to Glu$^{108}$) with a calculated molecular mass of $\sim 3.7$ kDa. This peptide would contain a few amino acids from the first tryptic photolabeled peptide and the complete sequence of the second. Other V8 peptides that contain the $^{125}$I-ASA-BZ-photolabeled amino acid (e.g. Leu$^{65}$) from the first tryptic peptide in fraction 45 would be too small to detect on SDS-PAGE and would migrate with the dye front.

The photolabeling of two tryptic peptides by $^{125}$I-ASA-BZ was of the same intensity as determined from the radiolabel associated with each peptide. Moreover, comparison of the amino acid sequences of the two $^{125}$I-ASA-BZ-photolabeled peptides showed no apparent sequence identity to support the possibility of two similar binding domains. Thus, the photoaffinity labeling of $\beta$-tubulin at two sites is likely due to rotational freedom about the ASA moiety that allows the photoreactive group in ASA-BZ to cross-link more than one sequence in native protein. In this respect, it was shown recently that two different sequences in $\beta$-tubulin are photolabeled by different photoreactive analogues of taxol (p-azidobenzoyl- or m-azidobenzoyltaxol; Refs. 34 and 52). The photolabeling of the two domains (i.e. amino acids 1–31 and 217–231) in $\beta$-tubulin by m-azidobenzoyl- and p-azidobenzoyltaxol is thought to be due to differences in the position of the photoreactive groups (52). Although further characterization of the taxol binding domain is required, in general, the photolabeling of several distant sites in a protein is compatible with the three-dimensional nature of a drug binding site. Consequently, it is conceivable that other photoreactive analogues of BZ could cross-link differ-
BZ-photolabeled peptides (N-terminal 63–103) of H. contortus to bovine or human β-tubulin show two different amino acid residues (Ala<sup>63</sup> → Gin and Leu<sup>86</sup> → Ile) that could confer weaker binding of BZ to mammalian tubulin. Alternatively, the observed safety of BZ as anthelmintics may be unrelated to BZ-tubulin binding but due to differences in metabolism or detoxification pathways. For example, the rapid and extensive metabolism of BZ into less toxic metabolites (e.g., sufoxides and sulfones) by the liver microsomal enzymes (56, 57) may account for some lack of host toxicity. Parasites, on the other hand, lack these metabolic pathways and are killed by BZ. In addition, we have shown recently that BZ are substrates for the P-glycoprotein transporter in multidrug-resistant tumor cells (5). It may be speculated that P-glycoprotein which is overexpressed in several normal tissues and organs (58, 59) could mediate the transport of BZ. Thus, P-glycoprotein rather than differences in tubulin amino acid sequence may mediate the observed safety of BZ to the host. The latter speculation is interesting since ivermectin, a potent anthelmintic agent, is also remarkably safe to the host (60), and its accumulation in normal tissues is affected by the presence of P-glycoprotein (61). The latter speculation is interesting since BZ-photolabeled peptides (N-terminal 63–103) of

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