Abstract
The cytochrome P450 family of enzymes is involved in the Phase I metabolism of a wide variety of compounds. Although generally involved with detoxification, overexpression of one family member, cytochrome P450 1B1 (CYP1B1), has been associated with human epithelial tumors. As such, CYP1B1 was hypothesized to be a novel target for the development of anticancer therapies. We investigated expression of CYP1B1 protein in 61 human colorectal adenocarcinomas and compared this to that observed in 14 histologically normal human large bowel samples removed from patients undergoing surgery for large bowel tumors. Although we confirmed that CYP1B1 was expressed at high levels in human colorectal tumor epithelia, we also found that CYP1B1 was not absent from normal colonic epithelia but was expressed at low levels. The expression of CYP1B1 in colon tumors does not correlate with tumor stage or degree of lymph node invasion in this study. Furthermore, in addition to expression in colon epithelia, CYP1B1 is also observed in blood vessels within the colon. As with the epithelia, levels of CYP1B1 were higher in tumor vasculature than that of the normal colon. Although these observations greatly support the development of CYP1B1 targeted anticancer therapies, they also indicate the caution that should be observed when developing such drugs.

Introduction
The CYP enzymes are a family of constitutive and inducible heme-containing mono-oxygenases (1) with central roles in the Phase I metabolism of xenobiotics. As such, the CYPs are important mediators for the toxicology and pharmacokinetics of many compounds to which man is exposed. Although CYPs normally act to detoxify putative carcinogens, recent reports have suggested that the CYP-mediated metabolism of certain xenobiotics may actually result in a more reactive and dangerous metabolite (2–5). This is the case for many polycyclic aromatic hydrocarbons activated by the CYP1 family (6, 7). Furthermore, several studies have demonstrated that the CYPs can activate or deactivate chemotherapeutic agents and as such influence the response of tumors to anticancer drug therapy (8–10). Therefore, the CYP enzymes have important roles in both tumor development and response to chemotherapy.

One family member that has recently gained a significant level of interest in tumor development and therapy is CYP1B1. Although the expression profile and activity of CYP1B1 have not been fully elucidated, mRNA expression has been reported in several tissues, including brain (11–13), kidney (13–15), and lung (16, 17). In contrast, endogenous CYP1B1 protein expression has been detected in very few normal human tissues. Of the human tissues demonstrated to be positive for CYP1B1 protein, the majority appear to be responsive to steroid hormones such as endometrium (18), mammary tissue (19, 20), testes, and prostate (21). Conversely and of importance for this study, overexpression of CYP1B1 has been demonstrated in a number of primary human tumors (8, 22–24). Accordingly, expression of CYP1B1 protein has been suggested to be tumor specific as a result of its high expression in tumors compared with normal tissues (24, 25). On the basis of this, differential CYP1B1 was suggested to be an ideal molecular target for the development of new anticancer drugs. Putative strategies may involve either inhibiting CYP1B1 activity (26) or using the metabolic activity of CYP1B1 to activate essentially nontoxic prodrugs to cytotoxic compounds (27, 28). Although previous studies have suggested CYP1B1 protein expression to be tumor specific (8, 22, 24, 25), these studies focused primarily on tumor epithelia and did not comprehensively address CYP1B1 expression in other tissue regions. In addition, other studies suggested a lack of differential between tumor and normal tissue (29). Therefore, more comprehensive studies and additional validation of CYP1B1 expression are required to assist the drug development proc-
Overexpression of CYP1B1 in Colon Adenocarcinomas

Mens, which were excised study also includes 14 normal human colonic tissue speci-
ple) or lymph node positive (22 samples). In addition, the stage 1 (pT1 and pT2; 14 samples) or stage 2 tumor (pT3 and statistical comparison, tumor stages were classified as either
dence of lymph node metastasis, and patient gender. To aid presented; Table 1 shows the distribution of tumor stage, evi-
was collected from patients undergoing resections for a

tissue. The presence of CYP1B1, its relationship to tumor

evidence of lymph node metastasis, and patient gender. To aid statistical comparison, tumor stages were classified as either

dess. In this study, we analyzed the expression of CYP1B1
prostate in human colorectal tumors and normal colorectal
tissue. The presence of CYP1B1, its relationship to tumor
stage, lymph node invasion, and its expression throughout the tissue were thoroughly examined. The overall aim of this study was to validate CYP1B1 as a target for new drug
design in human colon cancer.

Materials and Methods

Clinical Material. A total of 61 formalin-fixed, paraffin-
embedded specimens of human colorectal adenocarcinoma was collected from patients undergoing resections for a range of colorectal cancers. For inclusion in this study, we confirmed that none of the patients had received anticancer chemotherapy before surgery. All tumors were adenocarcinomas of colon or rectum with a range of stages represented; Table 1 shows the distribution of tumor stage, evidence of lymph node metastasis, and patient gender. To aid statistical comparison, tumor stages were classified as either stage 1 (pT1 and pT2; 14 samples) or stage 2 tumor (pT3 and pT4; 47 samples) and either lymph node negative (39 samples) or lymph node positive (22 samples). In addition, the study also includes 14 normal human colonic tissue specimens, which were excised ≥30 cm from the tumor. Nineteen of the colorectal tumor blocks and 5 normal colon blocks were used for construction of TMAs and all blocks were sectioned and used for conventional immunohistochemistry. All experiments were performed after first obtaining both consent from patients and the local research and ethics committee according to Medical Research Council regulations. Patient details were anonymized to ensure confidentiality.

Antibodies. Two different antibodies against CYP1B1 were used in this study: a rabbit polyclonal raised against a CYP1B1 peptide (Gentest Corporation, Woburn, MA); and a mouse monoclonal antibody (LDS101) raised against a human CYP1B1 peptide (a kind gift from Dr. Lesley Stanley, De Montfort University, Leicester, United Kingdom). Pericytes and blood vessels were identified using an α-smooth muscle actin immunohistochemistry kit (Sigma, Poole, United Kingdom).

<table>
<thead>
<tr>
<th>A. pT stage</th>
<th>Tumor stage</th>
<th>Percentage of total (61 samples)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>I</td>
<td>3.2% (2)</td>
</tr>
<tr>
<td>2</td>
<td>II</td>
<td>19.7% (12)</td>
</tr>
<tr>
<td>3</td>
<td>II</td>
<td>63.9% (39)</td>
</tr>
<tr>
<td>4</td>
<td>II</td>
<td>13.1% (8)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B. Lymph node metastasis</th>
<th>Percentage of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>64% (39)</td>
</tr>
<tr>
<td>Positive</td>
<td>36% (22)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>C. Sex</th>
<th>Percentage of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>46% (28)</td>
</tr>
<tr>
<td>Male</td>
<td>54% (33)</td>
</tr>
</tbody>
</table>

Table 1 Distribution of clinicopathological characteristics of human colon cancer: tumor stage, lymph node status, and patient gender

Data is shown as percentages (with actual patient numbers in parentheses).

TMA Construction. TMAs were constructed from 19 human colorectal cancer and 5 normal colon paraffin embedded blocks. Standard histopathological examinations and classifications were performed. TMA construction was achieved using a Beecher Instruments microarrayer (Silver Spring, MD) using a modified method of that by Bubendorf et al. (30). Briefly, sections of each paraffin-embedded donor block were stained using H&E, examined by microscopy and an area containing tissue of interest marked on the wax block. Cylindrical cores (600 μm) were taken from these representative areas and transferred into a recipient block. Tissue sampling used four cores from each tumor block to provide representative data on each parent block (31, 32). Normal mouse testis was used as an orientation control on each TMA block. Sections, 5-μm thick, were cut from the recipient TMA blocks and mounted on glass slides using a tape transfer system (Instrumedics, Hackensack, NJ). H&E staining for verification of histology and sample integrity was performed on the first section cut from each microarray block. TMA slides were then subject to immunohistochemical analyses.

Induction of CYP1B1 in MDA-MB-468 Cell Line. The MDA-MB-468 mammary adenocarcinoma cell line was provided by the National Cancer Institute (Frederick, MD). Cells were grown as monolayers in RPMI medium (Sigma) supplemented with 10% fetal bovine serum (Invitrogen, Paisley, United Kingdom) and 2 mM glutamine (Sigma) in a humidified atmosphere of 5% CO2 at 37°C. Expression of CYP1B1 was induced by adding benz(a)anthracene (10 μM final concentration; Sigma) to the tissue culture media for 24 h before harvesting for Western blotting.

Western Blotting. After treatment of MDA-MB-468 cells with or without benz(a)anthracene (10 μM), cells were scraped into lysis buffer [20 mM Tris-HCl (pH 7.4), 2 mM EDTA, 2 mM EGTA, 1% NP40, 1 mM phenylmethylsulfonyl fluoride, 1× protease inhibitor mixture (Sigma), 1× phosphatase inhibitor (Sigma), 1 mM DTTT) at 4°C. Cell lysates were sonicated (3 × 15 s, 4°C) and centrifuged at 10,000 × g for 10 min. The supernatants were recovered and their protein concentrations measured using Bio-Rad protein assay reagent. Recombinant CYP1A1, CYP1A2, CYP1B1 proteins (Gentest Corporation) were diluted in PBS to equivalent concentrations. Equal amounts of cell lysate protein (50 μg) and CYP protein (1 pmol) were resolved by SDS-PAGE (10%) and transferred to nitrocellulose membranes (Hybond-ECL; Amersham, Little Chalfont, United Kingdom). Membranes were subsequently blocked with 5% nonfat milk powder in TBS. Membranes were probed with anti-CYP1B1 polyclonal antibody [Gentest Corporation; 1/2000 v/v in TBS-Tween (TBS, 1% milk powder, 0.2% Tween 20 (Sigma)) for 90 min at room temperature. Unbound antibody was removed by washing in TBS and 0.1% Tween 20 (TBST). Membranes were incubated with horseradish peroxidase-linked anti-rabbit secondary antibody (Gentest Corporation) for 60 min at room temperature. Membranes were washed again in TBST. Antibody antibody complexes were detected by enhanced chemiluminescence (Amersham) and detected on X-ray film.

Immunohistochemistry. Formalin-fixed, paraffin-embedded tissue sections (5-μm thick) were dewaxed in xylene and rehy-
drated to water through graded alcohols. Endogenous peroxidase activity was quenched with freshly prepared 1% hydrogen peroxide for 30 min at room temperature. Nonspecific antibody binding was blocked in 5% normal goat serum (Vector Laboratories, Peterborough, CA) for 20 min at room temperature in a humidified atmosphere. Sections were then incubated with primary antibody: the sections were incubated for 90 min at room temperature in a humidified atmosphere, with the primary antibodies diluted either 1:750 (Gentest Corporation) or 1:1000 (LDS101) in PBS. After washing in PBS, sections were incubated for 30 min at room temperature with either biotinylated goat antirabbit or horse antimouse immunoglobulins (0.5% v/v; Vector Laboratories) in PBS, washed in PBS, followed by incubation with peroxidase-conjugated streptavidin label for 30 min. The resultant immune peroxidase activity was developed using 3,3′-diaminobenzidine according to the manufacturer’s instructions (Vector Laboratories). Immunohistochemistry for α-smooth muscle actin was performed using the Sigma immunohistology kit as directed (Sigma). Sections were counterstained with Harris’ hematoxylin and mounted in DPX mountant (Sigma). Appropriate negative controls were performed by omitting the primary antibody and/or substituting it with an irrelevant antiserum. Slides were examined using a Leica DMRB microscope fitted with a JVC video camera (Leica Microsystems, Milton Keynes, United Kingdom) and images collected using AcQuis Bio software (Syncroscopy, Cambridge, United Kingdom).

Stained sections were independently assessed by three observers. Specimens were scored for density and extent of CYP1B1 expression on a scale from 0 (negative) to 3 (strong staining). The expression profile of CYP1B1 throughout the section was analyzed and scored with respect to epithelia, blood vessels, and smooth muscle. For each histological section, a mean score was calculated for each tissue type.

Inhibition of CYP1B1 Staining. To validate immunohistochemical expression, CYP1B1 primary antibody (1:750; Gentest Corporation) was preincubated with an equal mass of CYP1B1 protein (1:100; Gentest Corporation) for 30 min at room temperature to block CYP1B1 antibody immunoreactivity. The antibody-protein mix was then used in place of primary antibody for immunohistochemistry, and the resultant staining compared with that observed without blocking protein.

Results

CYP1B1 Antibodies. Previous studies have demonstrated the induction of CYP1B1 protein expression in human breast adenocarcinoma cell lines by benz[a]anthracene (33). By Western blotting, a protein band of 60 kDa colocalizing with CYP1B1 protein was evident upon benz[a]anthracene treatment (Fig. 1A). No additional bands were evident on this blot, supporting the specificity of this antibody against CYP1B1. The antibody recognizes CYP1B1 but not the other CYP1 family proteins, CYP1A1 or CYP1A2 (Fig. 1B). Furthermore, this antibody does not recognize CYP3A4, which has recently been shown to be elevated in human colorectal cancer (Ref. 34; Fig. 1B).

Expression of CYP1B1 in Colorectal Cancer. CYP1B1 expression was examined using both histological sections and TMAs of human colorectal cancer and normal colon tissue (Figs. 2 and 3), and the staining intensity and distribution were scored on a scale from 0–3. The staining intensity observed in TMA cores was consistent with that observed in whole tissue sections supporting both the use of TMAs and eliminating interexperimental staining differences (data not shown). Verification of CYP1B1 expression was confirmed using an alternative anti-CYP1B1 antibody (Fig. 2, A and B) and by inhibition of staining using CYP1B1 protein as a blocking step (Fig. 2, C and D).

Epithelia. CYP1B1 was expressed cytoplasmically in colorectal epithelia (Fig. 3). A significant difference was observed between expression in tumor epithelia (1.66 ± 0.08) and expression in normal colorectal epithelia (0.24 ± 0.08; Table 2). No difference was observed between stage 1 tumors (1.66 ± 0.10) and stage 2 tumors (1.66 ± 0.06). Similarly, no significant difference was observed between those tumors demonstrating lymph node metastases (1.75 ± 0.09) and those without lymph node metastasis (1.61 ± 0.06; Table 2).

Blood Vessels. CYP1B1 expression was observed in blood vessel walls in both colorectal tumors and normal colorectal tissue (Fig. 2). A significant difference in expression was observed between blood vessels in normal colon (1.46 ± 0.14), stage 1 colon tumors (2.56 ± 0.09), and stage 2 tumors (2.13 ± 0.07; Table 2). No sex differences were observed in blood vessel expression (data not shown). Within tumors, expression of CYP1B1 was greater in blood vessels proximal to the tumor than those more distal (data not shown). CYP1B1 staining in blood vessels is localized primarily within pericytes, an observation additionally supported by the colocalization of CYP1B1 and α-smooth muscle actin (Fig. 2G).
Smooth Muscle. CYP1B1 antibody immunoreactivity was noted in the smooth muscle encapsulating the colon (muscularis propria) in both tumor and normal colorectal tissue. No apparent differences in staining were observed between normal colon, stage 1, or stage 2 colon tumors (data not shown). In contrast to other structures within the colon, preincubation of the antibody with CYP1B1 protein resulted in a ~50% reduction in staining rather than a complete abolishment as observed in the epithelia and blood vessels.

Discussion

This study has investigated the presence of CYP1B1 in a large number of resection specimens from patients with human colorectal tumors to confirm the hypothesis that CYP1B1 is up-regulated in tumors and as such a valid target for anticancer drug development. The data presented support and extend previous studies in which overexpression of CYP1B1 was demonstrated in many human tumors (24, 25,
In contrast, our observations did not support previous studies in which CYP1B1 protein was undetectable in normal human tissues (24, 25, 35). Although CYP1B1 was detected at high levels in human colon tumors (mean score = 1.66; \(n = 61\)), normal colon was also found to express CYP1B1, albeit at a much lower level (mean score = 0.24; \(n = 14\)). Our results demonstrate a previously unreported pattern of expression, which includes not only the epithelium of the large bowel, but also blood vessels and to a certain extent the smooth muscle. Taken together, these observations have strong implications for current chemotherapies as well as the development of novel and more efficient therapeutic strategies.

We initially concentrated on confirming the specificity of the antibody against human CYP1B1. By Western blotting, we confirmed that the antibody (Gentest Corporation) specifically detected CYP1B1 and neither CYP1A1, CYP1A2, or CYP3A4 and that no additional bands were evident in whole cell lysates of MDA-MB-468 cells in which CYP1B1 expression had been induced by benz[a]anthracene. Both the polyclonal (Gentest Corporation) and monoclonal (LDS101) antibodies gave an identical staining pattern and intensity of immunoreactivity on serial histological sections additionally supporting our observations as being CYP1B1. To additionally confirm antibody specificity, we used purified CYP1B1 as a blocking protein for immunohistochemistry. Inclusion of the blocking step resulted in abolishment of antibody staining in both the epithelia and blood vessels within the sections. However, preincubation with CYP1B1 protein did not completely abolish the observed staining in the smooth muscle. One explanation for this ob-

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Table 2  Summary table describing expression of CYP1B1 by immunohistochemistry

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Stage 1 tumor</th>
<th>Stage 2 tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>A*</td>
<td>0.24 ± 0.08</td>
<td>1.66 ± 0.10</td>
<td>1.66 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>Negative lymph node</td>
<td>Positive lymph node</td>
</tr>
<tr>
<td>B</td>
<td>0.23 ± 0.08</td>
<td>1.61 ± 0.06</td>
<td>1.75 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>Stage 1 tumor</td>
<td>Stage 2 tumor</td>
</tr>
<tr>
<td>C</td>
<td>1.47 ± 0.15</td>
<td>2.57 ± 0.06</td>
<td>2.13 ± 0.07</td>
</tr>
</tbody>
</table>

* A, normal colon, stage 1 (pT1 and pT2), and stage 2 (pT3, pT4) colorectal adenocarcinomas; B, normal colon, tumors without lymph node metastasis, and with lymph node metastasis; C, blood vessels of normal colon, stage 1 (pT1 and pT2), and stage 2 (pT3, pT4) colorectal adenocarcinomas.

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35). In contrast, our observations did not support previous studies in which CYP1B1 protein was undetectable in normal human tissues (24, 25, 35). Although CYP1B1 was detected at high levels in human colon tumors (mean score = 1.66; \(n = 61\)), normal colon was also found to express CYP1B1,
servation may be that a proportion of the smooth muscle staining is an artifact. This hypothesis is supported by a study investigating somatostatin receptors in the human gastrointestinal tract in which positive immunoreactivity was observed in smooth muscle cells was inhibited by antibody preabsorption but did not correlate with receptor autoradiography (36). On the basis of our antibody optimization results and observed expression in pericytes and blood vessels, a more plausible explanation is that although the amount of CYP1B1 protein was equal to the amount of antibody, this was insufficient to block all staining in the smooth muscle. The presence of CYP1B1 in smooth muscle is supported by other recent studies in which CYP1B1 protein was detected in murine aortic smooth muscle cells (37) and human vascular smooth muscle cells (38, 39).

To eliminate the possibility that differentials in CYP1B1 staining were a result of interexperimental variability, we also performed immunohistochemical analyses on a TMA containing representative samples from our study. Using this method, each sample is subjected to exactly the same treatment during the staining procedure. The same pattern of staining and expression profile were observed with the TMA as with the whole sections, additionally supporting our observations as being representative.

In this study, CYP1B1 expression was observed in colonic epithelia from both tumor and normal tissue. We detected CYP1B1 protein at a much higher level in colonic tumors than normal colonic tissue supporting CYP1B1 as a valid target for anticancer therapy. The level of CYP1B1 expression in stage 1 colon tumors (pT1 and pT2) was not significantly different from that of stage 2 colon tumors (pT3 and pT4), and no difference was observed between lymph node-negative and lymph node-positive tumors. In addition to supporting CYP1B1 as a chemotherapeutic target, these data suggest that up-regulation of CYP1B1 could be used as a diagnostic marker for colon cancer. Furthermore, these results also suggest that CYP1B1 does not have a direct role in tumor invasion or metastasis.

The expression of CYP1B1 in colorectal vasculature is a previously unreported observation with strong implications for drug development. CYP1B1 protein was evident in the pericytes surrounding blood vessels in both tumor and normal colorectal sections. In agreement with the epithelial results, expression in the blood vessels of the normal colon (1.46 ± 0.14) was much lower than that observed in either the stage 1 (2.56 ± 0.09) or stage 2 (2.13 ± 0.06) colonic tumors. Several hypotheses exist to explain the expression of CYP1B1 in normal colonic blood vessels such as exposure to dietary carcinogens (40, 41), tobacco smoke (17), or metabolism of an endogenous substrate. In addition, a recent study demonstrated that CYP1B1 was one of only two genes up-regulated in blood vessels in response to shear stress and laminar flow (42), a finding that is directly applicable to the in vivo situation and our observations. With reference to our results, although we observed CYP1B1 in normal vasculature, there is clearly an overexpression within the tumor environment. This differential in CYP1B1 levels, in addition to that observed in epithelia, strongly supports its use as a target for anticancer therapy but also highlights the caution that should be observed when developing such therapies.

We cannot, from these data, surmise that CYP1B1 up-regulation is an early event in colorectal tumorigenesis, although this has been suggested for both uterine (43) and breast (33, 44) tumorigenesis. In addition, a role for genetic polymorphisms of CYP1B1 in the susceptibility and pathogenesis of many tumor types has been demonstrated (45–52). To the best of our knowledge, such a study has yet to be undertaken for human colorectal cancer.

In summary, our findings that CYP1B1 protein is expressed at a much higher level in colorectal tumors than normal colonic tissue reinforces previous studies (24) and strongly supports CYP1B1 as a target for therapeutic exploitation. Conversely, the expression of CYP1B1 in normal colonic epithelia, blood vessels, and smooth muscle was previously unreported. Taken together, these observations have implications for drug development strategies and illustrate the need for analysis of not just the tumor epithelia but also the surrounding structures and comparative normal tissues. From a drug development perspective, the large differential in CYP1B1 expression between normal colon and colon tumors supports both the development of CYP1B1-activated prodrugs (10, 53) and their likely tumor-specific action. We speculate that this specificity will not be confined to malignant cells but will include relatively tumor-specific antivascular effects. This would represent, in one drug treatment, a combination of effects that is currently accomplished by multiple drug regimens.

Acknowledgments

We thank Dr. Lesley Stanley (De Montfort University, United Kingdom) for kindly providing antibodies and members of the Tom Connors Cancer Research Centre for critical reading of the manuscript.

References

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Molecular Cancer Therapeutics

Cytochrome P450 1B1 (CYP1B1) Is Overexpressed in Human Colon Adenocarcinomas Relative to Normal Colon: Implications for Drug Development

Paul Gibson, Jason H. Gill, Parveen A. Khan, et al.


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