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Identification of cytochrome P450 enzymes in human colorectal metastases and the surrounding liver: a proteomic approach

C.S. Lane [a], S. Nisar [a], W.J. Griffiths [a], B.J. Fuller [b], B.R. Davidson [b], J. Hewes [b], K.J. Welham [a,1], L.H. Patterson [a,*]

[a] *Department of Pharmaceutical and Biological Chemistry, The School of Pharmacy, University of London, 29/39 Brunswick Square, London WC1N 1AX, UK*

[b] *Department of Surgery, University College Medical School, London, UK*

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Abstract

We describe the direct identification of multiple cytochrome P450 (CYP) enzymes in healthy and cancerous tissue. CYPs in human liver colorectal metastases were compared with those in the surrounding liver using a mass spectrometry-based proteomic approach. Nano-scale reversed phase liquid chromatography combined with electrospray ionisation tandem mass spectrometry has been used to identify CYPs with no pre-selection of the proteins anticipated. Fourteen distinct CYP enzymes from the subfamilies 1A, 2A, 2B, 2C, 2D, 2E, 3A, 4A, 4F, 8B and 27A were positively identified; 13 in the liver samples and 12 in the tumour tissue. It was found that three of the colorectal metastases expressed essentially the same drug-metabolising pattern of CYPs as the surrounding liver, whilst three tumour samples from different individuals showed no CYP expression. This was likely in at least one case to be due to low sample mass. The CYP expression profile in an individual tumour is likely to be an important determinant in predicting the outcome of cancer chemotherapy.

Keywords: Cytochrome P450; Proteomics; Electrospray ionisation; Tandem mass spectrometry; Human liver; Colorectal metastasis; Chemotherapy

1. Introduction

Cytochrome P450 proteins (CYPs) in the liver are known to be of major importance to the fate of anticancer agents; however their expression and role in tumours has received little attention. The completion of the draft of the human genome revealed the presence of approximately 100 different CYP genes, of at least which 59 are thought to be expressed based on transcript identification [1]. CYP-mediated metabolism is generally viewed as a route to drug detoxification and increased elimination, although CYP activation of certain anticancer drugs, e.g., cyclophosphamide, dacarbazine and thiotepa, has long been known and the importance of this process as a way of targeting novel anticancer therapy is being explored [2]. CYPs are also responsible for reductive metabolism and can contribute to the activation of tumour hypoxia targeted cytotoxins [3]. The outcome of cancer therapeutic in part relies on the expression profile of an array of different CYP enzymes, especially in the liver, contributing to the metabolic fate of administered drugs. The heterogeneity of the human population means that a CYP profile in any one individual is unique due to a combination of genetic and environmental factors including the plethora of drugs used in treating disease. Determination of the CYP protein expression profile on an individual patient basis prior to cancer chemotherapy could provide important information regarding the fate of the selected drugs and

* Corresponding author. Tel.: +44-20-7753-5883; fax: +44-20-7753-596.
E-mail address: medchem@ulsop.ac.uk (L.H. Patterson).
[1] Present address: Department of Chemistry, University of Hull, Hull, UK.

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hence outcome of therapy. In addition, the relative activity of liver and tumour drug metabolism will have a major impact in determining therapeutic outcome and in the development of tumour-specific prodrugs. Traditional methods for the detection of CYPs rely on immunoblotting, activity assays and the detection of CYP mRNA. These techniques have significant limitations. Immunoblotting, whilst being very sensitive, relies on the availability of enzyme-specific antibodies. In addition, it is necessary to pre-select which CYPs are to be investigated, and to identify each enzyme in turn. Activity assays that are designed to interrogate the activity of a CYP enzyme invariably require multiple analysis techniques, since different assays must be developed for different target substrates; even then, they may not be totally enzyme-specific. Measurement at the expression level is fraught with uncertainty since the presence and abundance of a particular type of mRNA does not necessarily infer the presence and abundance of the corresponding protein [4–6]. Mass spectrometry provides an attractive approach for the analysis of expressed proteins, offering uniquely the ability to directly detect low levels of multiple proteins in a single analytical run. Generally, two-dimensional gel electrophoresis (2DE) followed by matrix assisted laser desorption/ionisation–time-of-flight (MALDI–TOF) mass spectrometry and peptide mass fingerprinting (PMF) has become a principal approach for the proteomic profiling of various *in vitro* and *in vivo* biological systems [7,8]. A major drawback of the 2DE approach is its low performance in the separation of membrane proteins including CYPs [9]. We have demonstrated the use of nano-scale reversed phase liquid chromatography (nano-LC), electrospray ionisation tandem mass spectrometry (ESI-MS/MS) in combination with one-dimensional sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) for the identification of CYPs in male and female rat liver [10]. The aim of the present study was to adopt a proteomic analysis of CYPs in tumour and liver samples, and to reveal its potential in the assessment of which CYP enzymes could contribute to the outcome of chemotherapy.

2. Materials and methods

2.1. Subjects

The protocol for this study was approved by the Ethics Committee of the Royal Free Hospital and University College School of Medicine. Informed patient consent for use of tissues was obtained in all cases. Tissues for the study were accessed from the resected and discarded masses of tumour and surrounding liver, which were removed as part of the surgical treatment for hepatic metastases arising from colon cancers.

2.2. Tissue sampling

Samples of normal liver and of tumour (approximately 0.5 g) were taken in duplicate, and immediately snap-frozen in liquid nitrogen for further processing. The normal liver tissues were taken from the region of the resected mass most distant from the tumour. In each case, the tumour tissues were easily identifiable as hard, white deposits of diameters of 2 cm or greater. The metastatic nature of the deposits was confirmed by histopathological examination.

2.3. Preparation of microsomes

Frozen samples were ground either in a percussion mortar and pestle, or in a tissue dismembrator (Mikro-Dismembrator U, B. Braun Biotech International, Melsungen, Germany). Powdered tissue was placed into an ice-cold glass homogeniser, along with approximately 1 ml of homogenisation buffer for every 0.1 g of tissue, and homogenised using a polytetrafluoroethylene-head pestle. The homogenisation buffer contained 0.25 M sucrose, 50 mM Tris–HCl, pH 7.2, 4 °C, 1 mM ethylene diamine tetraacetic acid (EDTA), 100 mM sodium chloride, 0.1 mM dithiothreitol (DTT), 0.1 mM benzamidine, 0.1 μg/ml soya-bean trypsin inhibitor (SBTI) and 0.1 mM phenymethylsulphonyl fluoride (PMSF). Microsomes were prepared using differential centrifugation as follows: an initial centrifugation at 2400*g* for 20 min was used to sediment the cell debris, nuclei and unbroken cells. The supernatant was centrifuged at 12 000*g* for 20 min at 4 °C. Supernatant from this step was centrifuged at 180 000*g* for 60 min at 4 °C. The resultant microsomal pellet was suspended in 0.1 M Tris–HCl, pH 7.4, containing 15% glycerol, 1 mM EDTA, 0.1 mM PMSF, 0.1 mM benzamidine, 0.1 μg/ml SBTI, 7 μM pepstatin, 5 μg/ml chymostatin, 10 μM leupeptin and 5 μg/ml aprotinin, and then recentrifuged at 180 000*g* for 1 h. The final pellet was resuspended in 0.1 M Tris–HCl, pH 7.4, containing 15% glycerol, 1 mM EDTA, 0.1 mM PMSF, 0.1 mM benzamidine, 0.1 μg/ml SBTI, 7 μM pepstatin, 5 μg/ml chymostatin, 10 μM leupeptin and 5 μg/ml aprotinin, and stored at −80 °C. The microsomal protein concentration was determined using the Bradford assay [11].

2.4. SDS–PAGE

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis was performed using standard methods on the Hoefer Mighty Small gel system (Amersham Pharmacia, Buckinghamshire, UK). Microsome samples were diluted with denaturing sample buffer to contain 0.1 M DTT, 0.3% SDS, 0.002% bromophenol blue, 5% glycerol and 8.3 mM Tris–HCl pH 6.8, and heated at 95 °C for 10 min. Microsomal protein (approximately 25 μg where

available) was resolved on a 10% acrylamide gel. Gels were stained with 0.2% Coomassie Brilliant Blue R-250 in 30% methanol and 10% acetic acid for 1 h, and destained for approximately 2 h with the same solvent. Gels were left in 35% ethanol containing 2% glycerol overnight.

2.5. In-gel tryptic digestion and peptide extraction

The molecular weight region on the SDS–PAGE gel between 48 and 62 kDa was divided into six approximately equal bands, and each band excised with a razor blade. Bands were washed in distilled water until the pH was neutral, and completely destained using 50 mM NH_4HCO_3 in 40% ethanol. Bands were cut into fine pieces to increase the surface area, then dried first with acetonitrile and then in a SpeedVac for 30 min. Digestion was carried out using sequencing grade modified trypsin (approximately 75 ng/μl) (Promega, Southampton, UK) in 25 mM NH_4HCO_3. Sufficient trypsin solution was added to swell the gel pieces, which were kept on ice for 30 min and then covered with 25 mM NH_4HCO_3 and incubated at 37 °C overnight. Peptides were extracted from the gel pieces, with ultrasonication, using sequential washings with a solution of 5% trifluoroacetic acid (TFA) in 50% acetonitrile. The extracts were combined and dried in a SpeedVac to complete dryness. The resulting peptide mixtures were stored at −80 °C and reconstituted in 12 μl of 0.1% TFA immediately prior to analysis.

2.6. Nano-LC–ESI-MS/MS

Nano-LC was performed using an LC Packings Ultimate Capillary HPLC system with FAMOS™ autosampler (Dionex, Camberley, Surrey, UK). A separate Ultimate Micropump was employed as a loading pump. Sample (1 μl) was injected via a sample loop (using 0.1% TFA in water as the carrier solvent) onto a 1 mm × 300 μm PepMap™ C18 guard column (5 μm, 100 Å) (LC Packings). The sample was washed with 0.1% TFA for 3.5 min on the guard column before being switched onto a 15 cm × 75 μm PepMap™ C18 column (3 μm, 100 Å) (LC Packings) equilibrated with 95% mobile phase A (5% acetonitrile containing 0.1% formic acid) and 5% mobile phase B (80% acetonitrile containing 0.1% formic acid), at a flow rate of 200 nl/min. Five minutes after sample injection, the proportion of mobile phase B was increased linearly to 50% over 25 min, and then stepped up to 95% and maintained at this level for 10 min (wash phase). The column was then re-equilibrated for 20 min with 95% mobile phase A, 5% mobile phase B. The column effluent was continuously directed into an LCQ^{duo} (ThermoElectron, Hemel Hempstead, UK) mass spectrometer fitted with a nano-ESI source and spectra were recorded. Mass spectrometer conditions were optimised using in-solution tryptic digests of purified recombinant human CYP enzymes 1A2, 2E1 and 3A4, obtained from PanVera (Madison, Wisconsin USA). ESI was performed under the following conditions: positive ionisation mode; spray voltage, 1.8 kV; capillary voltage, 28 V; capillary temperature, 180 °C; no sheath or auxiliary gas used. Data was collected in the full scan and data-dependent MS/MS modes; 3 microscans were performed, with the maximum ion injection time of 200 ms. In the full scan mode, ions were collected in the m/z range 400–2000. The MS/MS collision energy was set to 35%.

2.7. Protein identification

MS/MS spectra were searched using Sequest Browser software [12] against a human protein database containing approximately 196 000 entries, of which 55 were CYPs (obtained from National Centre for Biotechnology Information (NCBI), Bethesda, MD, USA, July 2003). The aim of the Sequest approach is to find the amino acid sequence in the database that, when fragmented, would give a spectrum that correlates most closely with the experimental MS/MS spectrum. Candidate sequences are found in the database on the basis of intact peptide mass, and the theoretical spectra predicted to result from the fragmentation of these candidate peptides are generated and compared with the experimental MS/MS spectrum. The final score assigned to each candidate amino acid sequence is the Xcorr, a measure of how well the theoretical spectrum cross-correlates to the observed spectrum. Proteins that were matched by two or more peptides with Xcorr values $\geqslant 2.5$ and ΔC_n values (the difference in the Xcorr values for the top two candidate amino acid sequences) $\geqslant 0.1$ were considered identified, provided that those peptides were unique to the protein in the database; identifications were confirmed by manual examination of the MS/MS spectra. For proteins that were identified, but did not match this criteria, the data was assessed manually. The following parameters were used when creating data files from raw data files to be used in the Sequest search: precursor mass tolerance 1.4 m/z; minimum number of different ions 15; maximum number of intermediate scans 25; minimum number of grouped scans 1; minimum total ion count 5×10^5, parent ion mass range 400–4000 Da. The following parameters were used when searching data files using Sequest: fragment ion tolerance 0 Da, peptide mass tolerance 1 Da, average mass; maximum number of internal cleavage sites 2.

3. Results

3.1. Validation of the method

Initially, we evaluated the performance of the SDS–PAGE nano-LC–ESI-MS/MS procedure for the

identification of CYPs by loading known amounts of purified recombinant human CYP enzymes 1A2, 2E1 and 3A4 onto the SDS–PAGE gel and proceeding through the analytical cycle to identify the proteins. It was found that CYPs could be successfully identified down to approximately 1 pmol loaded onto the gel (data not shown). A successful identification was defined as one in which two or more unique peptides were found with Sequest Xcorr values $\geqslant 2.5$, $\Delta C_n \geqslant 0.1$.

3.2. Identification of CYPs in liver and tumour microsomes

Liver samples and, where evaluable, corresponding colorectal metastatic deposits in the liver were investigated from six patients (Table 1). SDS–PAGE was used to separate microsomal proteins; the 48–62 kDa region can be seen to be rich in protein, especially for the liver microsomes (see Fig. 1). The concentration of protein in microsomes prepared from tumour sample 5T was insufficient for analysis. For each of the other samples, six bands of approximately equal size covering the molecular weight range of the CYPs (48–62 kDa) were cut out and subjected to in-gel digestion with trypsin. The resultant peptides were extracted and analysed by nano-LC–ESI-MS/MS. MS/MS spectra were submitted to the Sequest algorithm, which identifies the peptides present in a two-step process by matching their MS/MS spectra against *in silico* generated theoretical spectra from the database, then performing cross-correlation analysis. This is illustrated in Fig. 2, which shows the MS/MS spectrum of the doubly charged peptide of *m*/*z* 476.0. Sequest determined the amino acid sequence of the peptide to be YGLLILMK, from CYP enzyme 2E1.

Using this methodology, we have identified 14 distinct CYP enzymes, 13 from the liver samples and 12 from three of the tumours (Table 2). CYP enzymes 1A2, 2A6, 2C8, 2C9, 2E1, 3A4 and 4A11 were found in all of the livers and in the three tumours in which CYPs were detected. In addition, CYP4F2 was found in all of the livers and two of the tumours. Other CYP enzymes identified were 2B6, 2C19, 2D6, 4F11, 8B1 and 27A1. Fig. 3 shows the mean number of peptides identified in the liver and three of the tumour samples for each CYP enzyme detected. Three of the tumours were found to express the same pattern of CYPs as the livers, although generally fewer peptides were found in the tumours. Two of the tumour samples (2T and 4T) did not appear to express any CYP enzymes, which can be explained by the low tumour mass available for analysis and/or generally low protein content of the tumour (Table 1). In addition, sample 4T was morphologically necrotic. Regarding the drug treatment, in some patients, with agents that can act as inducers and inhibitors of CYP subfamily members, there was no evidence that this in-

Table 1
Patient details and microsomal protein concentrations for samples taken from patients with metastatic colorectal cancer of the liver

Sample	Age of patient (years)	Gender (M/F)	Drug history	Chemotherapy[a]	Mass of tissue used to prepare microsomes (g)	Microsomal protein concentration in tissue (mg/g)[b]
1T	66	M	Warfarin, amiodarone, lisinopril, fluvastatin, aspirin	None	1.6	1.7
1L					1.9	7.6
2T	42	F	None	5-Fluorouracil (5-FU), oxaliplatin	0.37	0.67
2L					1.5	9.7
3T	60	F	None	None	1.7	0.53
3L					1.8	7.0
4T[c]	79	F	Amlodipine, thyroxine, isosorbide mononitrate, ranitidine, aspirin	5-FU, oxaliplatin	1.1	0.29
4L					1.0	3.1
5T	62	M	None	None	0.28	0.05
5L					1.3	4.3
6T	69	F	Warfarin, fluoxetine, cyclizine	5-FU, oxaliplatin	1.1	1.1
6L					1.2	3.9

T, tumour tissue; L, liver tissue; M, male; F, female.
[a] Last treatment >38 days prior to tissue resection.
[b] Determined using the Bradford assay.
[c] Necrotic tumour.

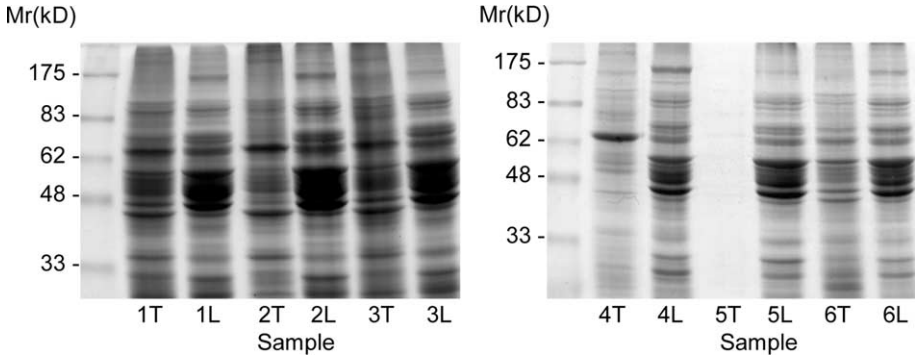

Fig. 1. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) gels showing microsomal protein from six pairs of liver and tumour samples. Molecular weight markers are indicated on the left (in kDa). Approximately 25 μg protein was loaded onto the gels for all samples except 5T (approximately 3 μg). The molecular weight region between 48 and 62 kDa was selected for analysis. For sample details, refer to Table 1.

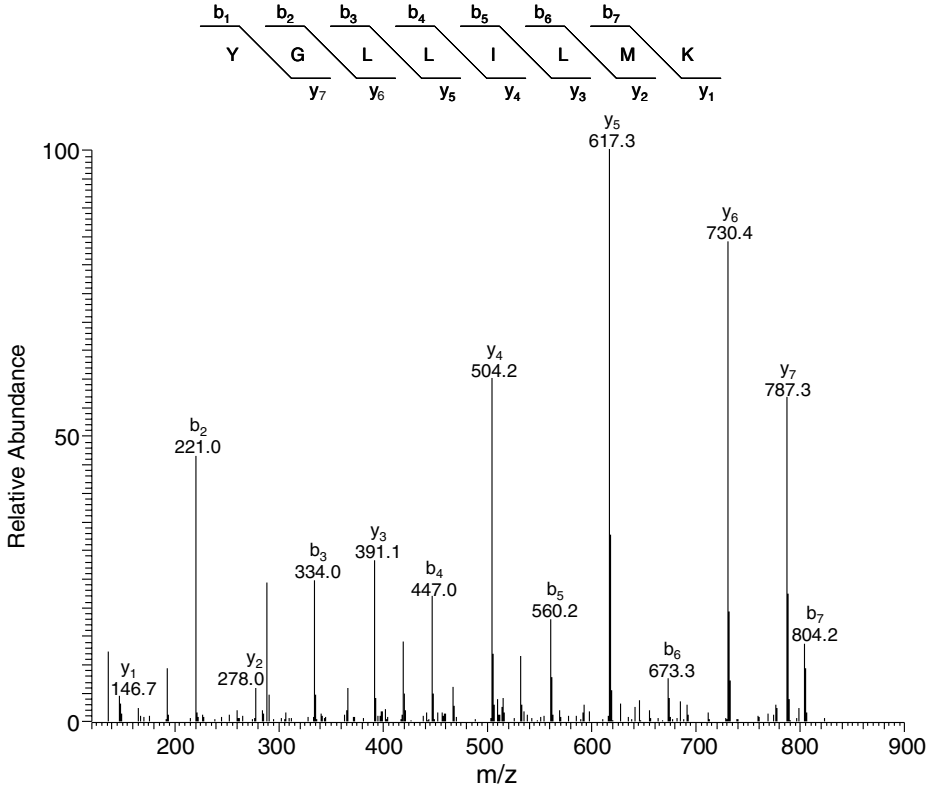

Fig. 2. MS/MS spectrum of the tryptic peptide YGLLILMK $[M+2H]^{2+}$ ion of m/z 476.0, identified to originate from CYP2E1. The amino acid sequence of the peptide is shown above the spectrum. y and b ions are formed by peptide bond cleavage with charge retention on the C-terminus and N-terminus, respectively.

fluenced the CYP expression profiles in the livers or tumours investigated (Table 1).

Although the subject of this study was the detection of CYPs, other proteins identified with high peptide numbers/sequence coverage included adenosine triphosphate (ATP) synthases and protein disulphide isomerases, which were present in all samples analysed, except for the necrotic tumour sample 4T, and flavin-containing monooxygenases and uridine diphosphate glycosyltransferases, which were identified in all samples analysed, except 2T and 4T (in which no CYP enzymes were identified).

4. Discussion

The importance of tumour CYP expression in influencing the outcome of chemotherapy through resistance

Table 2
CYP enzymes identified in the microsomal fractions of liver and tumour samples from six patients with metastatic colorectal cancer of the liver

Sample	CYP enzymes identified: number of matched peptides (% sequence coverage by amino acid)													
	1A2	2A6	2B6	2C8	2C9	2C19	2D6	2E1	3A4	4A11	4F2	4F11	8B1	27A1
1T	6 (16)	1[a] (3)	–	6 (14)	10 (29)	–	1 (3)	2 (3)	6 (16)	3 (8)	3 (8)	–	–	1 (5)
1L	7 (22)	11 (30)	1 (2)	10 (33)	12 (38)	–	1 (4)	6 (16)	7 (21)	6 (21)	6 (16)	–	–	–
2T	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2L	7 (18)	16 (42)	–	8 (28)	8 (24)	3 (9)	–	12 (31)	9 (25)	6 (17)	7 (16)	2[b] (6)	3 (8)	–
3T	1 (3)	5 (13)	–	4 (16)	4 (17)	2 (6)	–	6 (18)	5 (13)	2 (16)	1[c] (2)	1[c] (2)	–	–
3L	5 (14)	18 (47)	–	9 (30)	9 (29)	6 (18)	1 (4)	9 (24)	13 (36)	4 (10)	4 (10)	2 (5)	3 (11)	–
4T	–	–	–	–	–	–	–	–	–	–	–	–	–	–
4L	7 (17)	9 (24)	1 (2)	9 (30)	6 (22)	2 (6)	3 (15)	10 (25)	10 (31)	6 (14)	9 (20)	2 (6)	–	–
5T[d]	–	–	–	–	–	–	–	–	–	–	–	–	–	–
5L	11 (32)	19 (51)	1 (4)	12 (36)	9 (29)	3 (9)	–	10 (26)	15 (46)	7 (15)	5 (13)	2 (6)	1 (2)	–
6T	1 (3)	3 (8)	–	2 (6)	7 (21)	–	–	9 (24)	3 (8)	2 (5)	–	1[b] (4)	–	–
6L	1 (3)	12 (35)	–	7 (24)	10 (31)	4 (15)	1 (4)	4 (13)	8 (22)	4 (10)	11 (28)	1[b] (4)	–	–

T, tumour tissue; L, liver tissue; - indicates that the CYP was not identified.
[a] Peptide common to CYP2A6 and 2A13.
[b] Peptides could be from CYP4F8 and/or 4F11.
[c] Peptide common to CYP4F2, 4F3, 4F11 and 4F12.
[d] The microsomal protein concentration of sample 5T was too low for analysis.

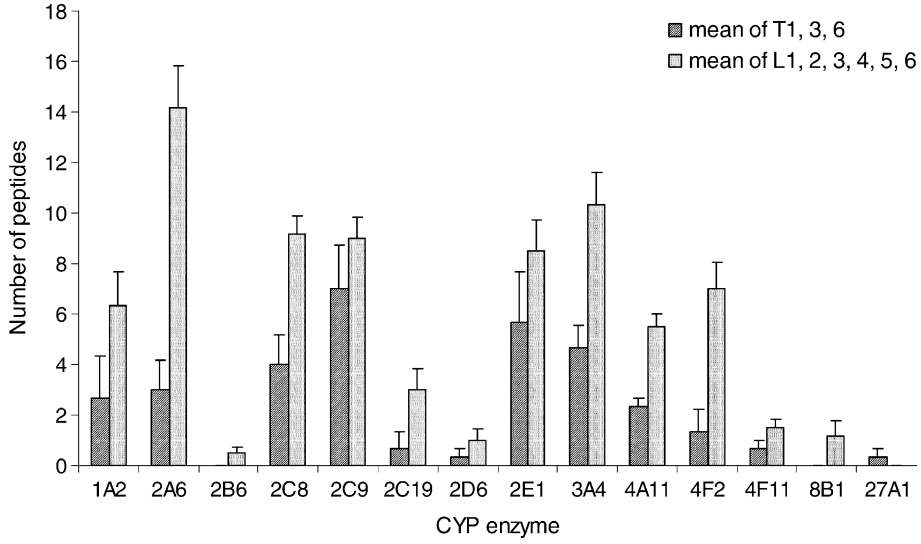

Fig. 3. The mean CYP content of six liver samples and three tumours from patients with metastatic colorectal cancer of the liver. Error bars show +1 standard error of the mean. No CYP enzymes were identified in samples T2, T4 or T5.

or prodrug activation is poorly understood [2]. Hence, there is a need to profile CYP expression in a way that does not rely on anticipating the CYPs present. This is the first study that investigates CYP enzymes in cancer without pre-selection of the proteins to be interrogated. In total, 14 CYP proteins were identified in colorectal metastases of human liver and matched liver samples. The technique employed one dimensional SDS-PAGE separation of the membrane bound CYPs, in-gel tryptic digestion and LC–ESI-MSMS separation and identification. This MS technique is preferred over MALDI–TOF and PMF when proteins are present in low abundance or as part of complex mixtures. The multiple CYP proteins identified include most of the major CYPs involved in drug metabolism (CYP1A2, 2A6, 2B6, 2C8 2C9, 2C19, 2D6, 2E1, 3A4) and those involved in bile acid synthesis and fatty acid metabolism (CYP4A11, 4F2, 4F11, 8B1 and 27A1). It is intriguing that essentially the same CYP profile was identified in both the liver and three of the tumour samples given that they originated in the colon. The metastases were shown to be free from liver tissue by histological examination, therefore this similarity cannot be caused by hepatocyte contamination. The possible influence of the liver environment on the CYP expression profile of deposited tumours invites further investigation.

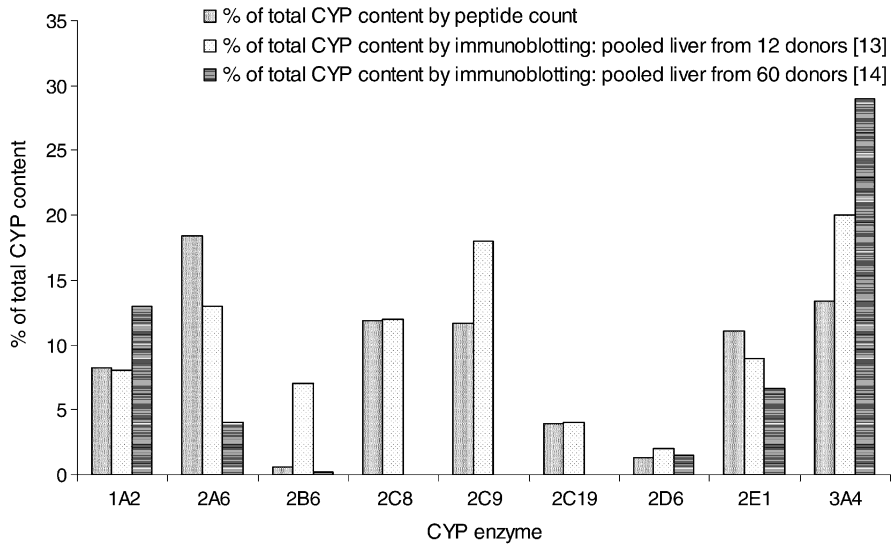

Fig. 4. The contribution of different CYP enzymes to the total CYP content in liver: comparison with published studies [13,14]. The % of total CYP content by peptide count was calculated using the average number of peptides for each CYP enzyme found in the liver samples (shown in Fig. 3), taking the total number of CYP peptides as 100%.

The contribution of each CYP isoform to the total CYP content in human liver has been investigated previously by immunochemistry [13,14]. The data of the current study are of a qualitative nature; nevertheless, the relative expression of CYPs based on the average number of peptides detected in the six liver samples in this MS approach compares favourably with previous accounts that have measured CYP expression by immunoblotting (see Fig. 4). This indicates that, to a first approximation, the number of tryptic peptides identified to originate from a given protein can be correlated to the expression level of that protein. A widely used method for relative quantitation of proteins with mass spectrometry involves the use of isotope-coded affinity tags (ICAT) [15]. With the ICAT approach, proteins isolated from different sources are differentially stable-isotope labelled, combined, digested, and then analysed by LC-MS/MS. This approach requires dilution of one sample by the other and is restricted to the analysis of cysteine-containing peptides. Such a procedure would inherently limit differentiation between CYP enzymes with high sequence similarity, and may also elevate the CYP protein detection limit.

In conclusion, the use of nano-LC–ESI-MS/MS for the study of CYPs in metastatic tumour and liver samples provides an attractive approach over traditional methods, offering uniquely the ability to directly detect multiple CYP enzymes without pre-selection. The CYP profile of the tumour samples demonstrates that colon metastases in the liver potentially have extensive drug-metabolising capabilities. Functional studies are required to investigate this further. The presence, or otherwise, of metabolically active CYPs in a colon metastastic deposit is likely to be important in determining the metabolic fate of chemotherapeutic agents and hence the outcome of treatment.

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