V79 Chinese Hamster Cells Genetically Engineered for Polymorphic Cytochrome P450 2D6 and their Predictive Value for Humans

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Summary

*With more than 30 genetic variants human cytochrome P450 2D6 (CYP2D6) presents the most extensive variation among all cytochromes P450. At the same time, roughly 30% of all drugs are metabolised by CYP2D6. Therefore, V79 Chinese hamster cells were genetically engineered for the genetic variants *1, *2, *9, *10, and *17 encoding active enzymes. These cells are to be used to understand and to predict variant-dependent metabolism of drugs and drug candidates. The V79-derived cell lines were extensively characterised for stable expression of mRNA, for enzyme activity using bufuralol hydroxylation, for CYP content by CO difference spectra, and for protein distribution and cellular location by in situ immunofluorescence. Based on these results, CYP-mediated metabolism of tamoxifen was investigated.*

Zusammenfassung: Gentechnologisch veränderte V79 Zellen des Chinesischen Hamsters für die polymorphen Cytochrome P450 2D6 und ihr prädiktiver Wert für den Menschen

*Mit mehr als 30 genetischen Varianten ist das humane Cytochrom P450 2D6 (CYP2D6) das variabelste aller Cytochrome P450. Gleichzeitig werden circa 30% aller Arzneimittel über CYP2D6 verstoffwechselt. Wegen dieser Bedeutung wurden V79 Chinesische Hamsterzellen gentechnologisch konstruiert, um die genetischen Varianten *1, *2, *9, *10 und *17, die für enzymatisch aktive Formen kodieren, stabil zu exprimieren und somit über ein Werkzeug zu verfügen, um genetisch abhängigen Metabolismus von Arzneimitteln und Arzneimittelkandidaten zu verstehen und vorherzusagen. Die Zelllinien wurden intensiv charakterisiert hinsichtlich der stabilen Expression der mRNA; der Enzymaktivität, gemessen an der Hydroxylierung von Bufuralol als Marker-Reaktion; des CYP-Gehaltes, ermittelt durch ein CO-Differenzspektrum; und hinsichtlich der zellulären Verteilung und Lokalisation durch in situ Immunfluoreszenz. Auf diesen Charakterisierungen basierend wurde der CYP-vermittelte Stoffwechsel von Tamoxifen untersucht.*

Keywords: cytochrome P450 2D6, V79 Chinese hamster cells, polymorphism, bufuralol, tamoxifen

1 Introduction

It is estimated, that at least 20% of all drugs currently prescribed are metabolised with extensive variation as a result of different pharmacogenetic backgrounds (Blech, 1999).

The intrinsic problems of drugs regarding genetic variation became evident by the late 70s, when sparteine (Eichelbaum et al., 1979) and debrisoquine (Evans et al., 1980) were found to have variable efficacy due to individual variations in metabolic efficiency. This observation was linked to a variation in the CYP2D6 gene (Gonzalez et al., 1988). Today, more than 30 genetic variants of CYP2D6 have been described, of which at least 5 code for active enzymes: *1 (wild type), *2, *9, *10, and *17 (Daly et al., 1996).

These genetic variations should be taken into account when evaluating enzyme kinetics. Genetically defined human liver samples could be used for these studies. However, these studies would be limited by the availability of human liver samples and by the complexity of the system due to the multitude of CYPs and overlapping substrate specificities. Furthermore, no animal model is available which would reflect the human CYP2D6 situation accurately. Only a rough distinction between fast and slow metabolisers has been described for inbred rat strains (Gonzalez et al., 1987).

A solution to circumvent these problems has been demonstrated with genetically engineered V79 Chinese hamster cell lines in recent years for several CYPs from different species, including humans, making up the V79 Cell Battery™ (Doehmer, 2001). Based on this experience, the collection of V79 cell lines was expanded to the expression of

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the relevant genetic variants of CYP2D6 in order to investigate and to measure enzyme kinetics for drugs metabolised by CYP2D6 variants. V79 cells are perfect host cells to express CYPs heterologously for several reasons. Most importantly, these cells do not express any measurable endogenous CYPs, providing a clean background and making the genetically engineered V79 cell a specific tool for the CYP isoform encoded in the cDNA stably integrated into the genome of these cells. Second, the protocol for making these cell lines makes it likely that only one active copy of a given cDNA is integrated, yielding expression and activity levels which are close to the native situation. This is important to allow direct extrapolation with a high predictive value for humans. Overexpression, as has been described for many other expression systems, was avoided on purpose in order to mimic physiological conditions. Therefore, genetically engineered CYP expressing V79 cells provide for work with live cells allowing for a cellular integrative linkage to all toxicological endpoints that can be measured in V79 cells.

The first "R", replacement, is experimentally the most demanding of the "3Rs", replacement, reduction, and refinement, because it requires the intelligent application of technology to yield an *in vitro* system which is superior over an animal model, and because it demands a predictive value for humans. Reduction and refinement cannot improve the situation, as uncertainty of the applicability to humans remains an intrinsic problem with any animal model.

2 Materials and methods

2.1 Chemicals

Bufuralol-hydrochloride and 1'-hydroxy-bufuralol-oxalate were kindly provided by Hoffmann-LaRoche, Basel, Switzerland. Z-4-hydroxy-tamoxifen and E/Z-4-hydroxy-tamoxifen was from RBI, Natick, Massachusetts, U.S.A., Z-tamoxifen from Sigma, Deisenhofen, Germany. E-tamoxifen, Z-N-desmethyl-tamoxifen-hydrochloride, and Z-N-didesmethyl-tamoxifen-hydrochloride, tamoxifen-1,2-epoxide, were kindly provided by Fujisawa (formerly Klinge Pharma), Munich, Germany. Z-tamoxifen-N-oxid and Z-4-hydroxy-tamoxifen-N-oxide were synthesised by one of us (T. E. M.).

All other chemicals were from Merck, Darmstadt, Germany, Riedel-de-Haen, Selze, Germany, Sigma, Deisenhofen, Germany, Gibco BRL, Eggenstein, Germany, Biochrom, Berlin, Germany, Serva, Heidelberg, Germany.

2.2 Cell culture

The V79MZ Chinese hamster cell line served as parental and host cell (Glatt et al., 1987).

Cells were maintained in DMEM supplemented with 10% fetal calf serum, 1 mM sodium-pyruvate, 4 mM L-glutamin, 100 U/ml penicillin and 100 µg/ml streptomycin and grown at 37°C, 7% CO_2, 90% humidity. For passage, cells were washed twice with PBS followed by incubation at 37°C in 0.05% trypsin/0.02% EDTA. Cells were passaged every second or third day to keep cells between 10% and 90% confluency.

Genetically engineered V79 cell lines were maintained under the same conditions except for the addition of 400 µg/ml G418, Gibco BRL, to the medium for selection.

2.3 Recombinant methods

Vectors: The CYP2D6 recombinant plasmid (Fig. 1) was based on pSV450 (Doehmer et al., 1988). For construction purposes the following vectors were applied: M13mp19 and pBluescript SK(+) (Stratagene, Heidelberg, Germany); pcDNA3.1Hygro(+), pRc/RSV, and pVL1393 (Invitrogen, Carlsbad, U.S.A.); pIC19H (ATCC, Manassas, U.S.A.), pSV2 neo (Clontech Laboratories, Palo Alto, U.S.A.).

CYP2D6-cDNAs: cDNAs for the allele hCYP2D6*1 in pBluescript SK(+), hCYP2D6*2 in pVL1393, hCYP2D6*9 in M13mp19 and hCYP2D6*10A in M13mp19, were provided by one author (U.M.Z). hCYP2D6*17 was reconstructed from *1 and *2 by PCR reactions.

Primers: All primers were synthesised by U. Linzner, GSF-National Research Center, Neuherberg, Germany. All primers with their position and orientation are shown in Fig. 5.

15889:
5´-GGAATGTCCTCTCAAGTAGA-3´
16014:
5´-CAGAGGTTTTCACCGTCATC-3´
16093:
5´-CATACTGCTTCGACCAGTTGCG-3´
16094:
5´-AGACGTGAAGCTTGCCGCCAC-CATGGGGCTA-3´
16095:
5´-CAGGACGTAGAATGGATCTGGAT-GATGGGCAC-3´
17606:
5´-GCAGGTGAGGGAGGCGATCAC-3´
20261:
5´-TATTCCAGAAGTAGTGAGG-3´
20262:
5´-ATCACCGAGCTGAGAAGC-3´

Sequencing: All constructs and the integrated genomic DNA were sequenced (SequiServe, Vaterstetten, Germany).

Polymerase Chain Reactions:

PCR#1: Insertion of mutation $C_{1111}T$ into the hCYP2D6*1-cDNA:
Starting reaction: primer: 1.4 µl 16094 (25 mM), 1.4 µl 16095 (25 mM); template: 1 µl pSV450h2D6*1 (15.5 ng/µl); 1 µl dNTPs (20 mM); 0.125 µl Red Hot DNA polymerase (5 U/µl, Advanced Biotechnologies Ltd., Sur-

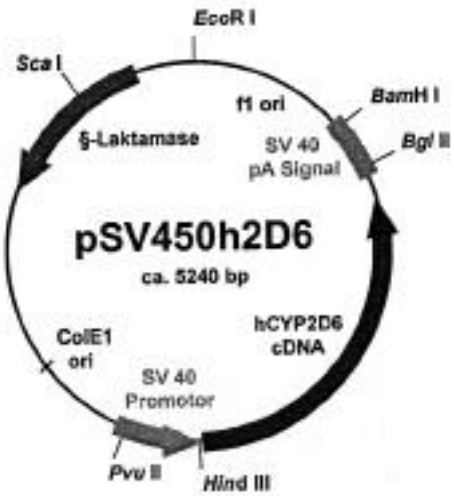

Fig. 1: Expression vector pSV450h2D6.

rey, England); 2.5 µl reaction buffer IV (10x); 1.5 µl magnesium chloride (25 mM); ad 25 µl H20; 1 drop of silicon oil
Equipment: Genius (Techne Ltd., Duxford Cambridge, England)
Programme: 1 min 94°C (1x); 1 min 94°C, 2 min 55°C, 3 min 72°C (30x); 10 min 72°C (1x)
Amplified product: 362 bp

PCR#2: Control-PCR for final ligation of the cDNAs into the vectors pSV450, pSV450HB, pSV450HK and pSV450HS:
Starting reaction: primer: 1.4 µl 20261 (25 mM), 1.4 µl 20262 (25 mM); template: 2 µl *E. coli* suspension; 1 µl dNTPs (20 mM); 0.2 µl *Taq* DNA polymerase (5 U/µl, QIAGEN, Hilden, Germany); 2.5 µl PCR-buffer (10x); ad 25 µl H_20; 1 drop of silicon oil
Equipment: Gene Amp PCR System 2400 (Perkin Elmer, Norwalk, CT, USA)
Program: 3 min 94°C (1x); 0.5 min 94°C, 1 min 56°C, 2 min 72°C (30x); 10 min 72°C (1x)
Amplified product: In case pSV450 contains an insert between restriction sites *Hin*d III and *Bgl* II, the length of the product is 72 bp + insert-bp. In case there is no insert, the product is a 72 bp fragment only.

PCR#3: Amplification of the stably integrated pSV450-expression cassette as isolated from genomic DNA of the recombinant V79MZh2D6-cell lines by *touch down*-PCR:
Starting reaction: primer: 1.4 µl 16014 (25 mM), 1.4 µl 15889 (25 mM); template: 1 µl genomic DNA (approx. 0.2 µg/µl); 1 µl dNTPs (20 mM); 0.5 µl *Taq* DNA polymerase
(5 U/µl, QIAGEN, Hilden, Germany); 2.5 µl PCR-buffer (10x); 5 µl Q-solution (5x); ad 25 µl H_20
Equipment: Gene Amp PCR System 2400 (Perkin Elmer, Norwalk, CT, USA)
Program: 1 min 94°C (1x); 1 min 94°C, 1 min 62°C → 52°C, 3 min
72°C (10x, annealing temperature increasing in 1°C-steps); 1 min 94°C, 1 min
52°C, 3 min 72°C (35x); 10 min 72°C (1x)
Amplified product: Depending on the integrated hCYP2D6-cDNA, a 2198 or a 2299 bp fragment is generated. In case of no insertion (mock transfection), the amplified fragment has a length of 700 bp only.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR): Reverse transcription and amplification of the heterologously expressed hCYP2D6 mRNA isolated from the recombinant V79MZh2D6-cell lines: following the Access RT-PCR System (Promega Corp., Madison, WI, USA).
Starting reaction: primer: 2 µl 16093 (25 mM), 2 µl 17606 (25 mM); template: 0.5 µl of total RNA (1-2 µg/µl); 1 µl dNTPs (10 mM); 1 µl AMV reverse transcriptase (5 U/µl); 1 µl Tfl DNA polymerase (5 U/µl); 10 µl AMV/Tfl reaction buffer (5x); 2 µl $MgSO_4$ (25 mM); ad 50 µl DEPC-treated H_20

Equipment: Uno-Thermoblock (Biometra biomedizinische Analytik GmbH, Göttingen, Germany)
Program: 50 min 48°C (1x); 2 min 94°C (1x); 1 min 94°C, 1 min 60°C, 2 min 70°C (40x); 10 min 70°C (1x)
Amplified product: A 410 bp fragment is generated.

Preparation, amplification, and isolation of plasmids:
Vector and insert were mixed at a ratio of 1:3 and ligated using 1 U of T_4 DNA ligase (Gibco BRL) and addition of 2 µl ligation buffer (5x) ad 10 µl total volume. After incubation at room temperature, the mixture was directly used for transformation of competent *E. coli* strains DH5α, HB101, JM109 or GM2167. Bacterial cells were made competent by starting a culture of 50 ml LB medium with a 1:100 dilution of an o/n culture, which was incubated at 37°C and 250 rpm on a shaker, reaching a density of 0.6 at OD 600 nm. Cells were harvested for 10 min at 800 x g at 4°C. Pelleted cells were resuspended in 2 ml TSS solution containing 10% (w/v) PEG 6000, 5% (v/v) DMSO, 50 mM $MgSO_4$ in LB medium. Aliquots were taken and stored at –80°C until use. Frozen aliquots were thawed on ice. Ligation mix was added and incubated for another 20 min on ice followed by a heat shock for 30 - 45 sec at 42°C in a water bath. A volume of 1.8 ml of LB medium and 36 µl 20% (w/v) glucose was added followed by incubation at 37°C and 250 rpm for 1 hour. Cells were harvested at 800 x g. The pellet was resuspended in 100 µl LB broth and plated on ampicillin containing agar plates, which were incubated at 37°C for 3 days. Plasmid DNA was prepared from selected colonies, characterised and used for transfection into V79MZ cells.

2.4 Construction of recombinant CYP2D6 expression vectors pSV450h2D6*1, *2, *9, *10, and *17

The cDNA encoding hCYP2D6*1 was isolated from pBluescript SK(+), hCYP2D6*9 and hCYP2D6*10A from M13mp19 by restriction with *Bam*H1 and *Eco*R1 and inserted into *Bam*H1/*Eco*R1 restricted plasmid pIC19H, from which the cDNAs were isolated by restriction with *Hin*dIII/*Bgl*II for insertion into the *Hin*dIII/*Bgl*II restricted pSV450. The hCYP2D6*2 cDNA was isolated from pVL1395 by restriction with *Hin*dIII and *Kpn*I and inserted into the *Hin*dIII/*Kpn*I restricted pICh2D6*10 from which it was recovered as a *Hin*dIII/*Bgl*II fragment and which was subsequently inserted into the *Hin*dIII/*Bgl*II restricted pSV450. The cDNA encoding hCYP2D6*17 was constructed by changing the 5´-end up to the *Xho*II site of the cDNA encoding hCYP2D6*2 by a PCR generated fragment already containing the *17 specific mutation $C_{1111}T$ (Fig. 2). The PCR fragment was generated using the primers 16094 and 16095 and *1 as template (PCR#1).

2.5 Transfection of V79MZ cells

The calcium/phosphate co-precipitation procedure was used for all transfections (Doehmer et al., 1998; Graham and Van der Eb, 1973; Parker and Stark, 1979). As much as 30 µg ScaI-linearised pSV450h2D6*1-, *2-, *9-, *10-, or *17- and 1 µg *Eco*RI-linearised pSV2neo-DNA were used in each transfection assay. Transfected cells were transferred to culture plates. Selection of cells was started 3 days later by the addition of 800 µg G418/ml DMEM medium. A mock transfected cell line was generated with

the selective marker pSV2neo only. G418 resistant colonies appeared 14 days after plating. Individual colonies were picked and checked for the integrated cDNA, expression, and enzyme activity. Selected colonies were observed for stability over a period of 3 months accompanied by repeated checking by *in situ* immunofluorescence.

2.6 Protein analytical methods

The presence of the correct CYP was checked by different methods.

***In situ* immunofluorescence:** Up to 10^4 cells of each selected clone were plated on micro chamber plates (Nunc, Naperville, U.S.A.) and cultivated o/n. Latex sealed chambers were stripped and cells were washed in PBS and thereafter fixed in ice-cold methanol/aceton (1:1) for 7 min and air dried. Fixed cells were incubated with 150 µl of the polyclonal CYP2D6 antibody 637.2 from rabbit prepared by one of us (U.M.Z) diluted 1:200 in culture medium and incubated for 90 min at room temperature, followed by 3 cycles of washing with PBS and incubation for 1 hour at room temperature in the dark with a secondary anti-rabbit IgG antibody coupled to FITC (Dianova, Hamburg, Germany) diluted 1:125 in culture medium followed by 3 cycles of washing, 10 min each, with PBS. Fixed and stained cells were covered with 100 µl of p-phenylendiammoniumdichloride solution (100 mg in 10 ml PBS and 80 ml glycerin) to prevent fading. Cells were observed and documented with the fluorescence microscope Axioplan (Zeiss, Oberkochen, Germany) at an excitation wavelength between 450 and 490 nm. For subcellular location of the cytochrome P450, cells were submitted to laser scanning microscopy (LSM 4.10; Zeiss, Oberkochen, Germany) at an excitation wavelength of 488 nm and emission at 515 and 565 nm.

Protein content: Total protein in the cell homogenate was determined according to Lowry et al. (1951) with BSA as standard.

SDS-polyacrylamide-gel-electrophoresis (SDS-PAGE): SDS-PAGE was applied to separate proteins in the cell homogenate by molecular weight (Laemmli, 1970) on Penguin P10DS (peqLab Biotechnologie GmbH, Erlangen) and Mighty Small SE245 (Hoefer Scientific Instruments, San Francisco, U.S.A.). Up to 5 µg of total protein was denatured in Laemmli-buffer (10% glycerin, 0.8% 2-mercaptoethanol, 10% upper Tris (4x: 0.5 M Tris, 8 mM EDTA, 0.4% SDS, pH 6.8), 8% SDS, 0.1% bromophenolblue, 0.1% xylenecyanol) for 10 min at 95°C and transferred to an 8% gel (4 ml 40% acrylamide/bisacrylamide (29/1), 5 ml upper Tris (4x), 11 ml bidistilled water, 12.5 µl TEMED, 150 µl 20% (m/v) APS). Proteins were separated on a 12% gel (9 ml 40% acrylamide/bisacrylamide (29/1), 7.5 ml lower Tris (4x: 2.5 M Tris, 8 mM EDTA, 0.4% SDS, pH 8.8), 13.5 ml bidestilled water, 13 µl TEMED, 100 µl 20% (m/v) APS) at 100 - 200 V in 1x electrophoresis buffer (50 mM Tris, 80 mM glycine, 2 mM EDTA, 1% SDS, pH 8.8). The *"Rainbow-Marker"* (14.3-200 kDa; Amersham, Little Chalfont, England) served as standard and for size estimation.

Western blotting: Proteins separated by SDS-PAGE were blotted for 30 min on Immobilon-P-Membran (Millipore, Dreieich, Germany) by the "*semi-dry*"-procedure at 225 mA in the "*Semi Dry* Electroblotter"-apparatus (Sartorius, Göttingen, Germany) according to Burnette, 1981. For immunodetection of hCYP2D6 the blotted membran was incubated o/n in PBS, substituted with 7% dry milk powder ("*Glücksklee*", Nestlé Deutschland AG, Frankfurt, Germany) at 4°C, briefly washed with PBS and soaked for 1 hour in the primary antibody solution (polyclonal anti-hCYP2D6 antiserum 637.2 from rabbit, diluted 1:100 in PBS), followed by 3 cycles, 15 min each, of washing in PBS and 0.5% Tween20, and further incubation for 30 min in the secondary antibody solution (POD-coupled anti-rabbit IgG from goat, 0.2 U/ml, diluted 1:10000 in PBS) and subsequently washed 3 times, 15 min each, in PBS, 0.5% Tween20. hCYP2D6 was detected with the ECL-Kit (*Enhanced Chemiluminescence*, Amersham, Little Chalfont, England).

Carbon monoxide difference spectra: The binding of carbon monoxide to iron is conventionally applied for the detection and quantitation of cytochrome P450. The binding is very specific for cytochrome P450 and allows for differentiation of cytochromes P450 from mitochondrial cytochrome oxidase, because the carbon monoxide complex of its reduced form absorbs light strongly at 450 nm, thus the name. A 1 ml aliquot of cell homogenate was thawed on ice and, after addition of 20 µl 100 mM PMSF in isopropanol, was carefully resuspended in 1 ml of solubilisation buffer (100 mM sodium hydrogenphosphate, pH 7.4, 10% (v/v) glycerin, 0.5% (w/v) emulgen 913). For stabilisation of CYP2D6 10 µl of 2 mM quinidin was added. The membrane bound CYP is released from the membrane by slow stirring on ice for 15 minutes and cleared from undissolved material by centrifugation for 10 min at 17,000 x g and 4°C (Evert et al., 1997; Tyndale et al., 1991). The supernatant was transferred to a dounce potter and reduced with a few crystals of sodiumdithionite during 15 strokes followed by transfer and split into 2 quarz glass cuvettes. A spectrum was recorded with the spectrophotometer Aminco DW-2000 UV/VIS-spectrophotometer (SLM Instruments Inc., Urbana, U.S.A.). The reduced spectrum was recorded between 400 and 500 nm. The samples were then mixed with 60 bubbles of CO for saturation and immediately measured for CO/reduced spectrum (Estabrook et al., 1972). The difference spectrum was recorded for both spectra. CYP concentration was calculated based on this difference spectra by applying the molar extinction coefficient of 91 $mM^{-1}cm^{-1}$ for the peak at 450 nm, respectively 110 $mM^{-1}cm^{-1}$ for the non-heme containing cytochrome P450 at 420 nm (Omura and Sato, 1964).

2.7 Enzyme activities

To measure hCYP2D6 activity the specific 1´-hydroxylation of (+)-bufuralol or the 4-hydroxylation of (-)-bufuralol was determined. A diluted aliquot of cell homogenate was thawed on ice. The reaction was started by the addition of homogenate to 100 µl total volume of the reaction mixture containing 150 µg of total protein (except V79MZh2D6*10: 300 µg), 200 µM bufuralol, 2 mM NADPH in 0.1 M potassium phosphate

buffer (pH 7.4) and was stopped by the addition of 12 µl 60% (v/v) perchloric acid after 30 min incubation in a water-bath at 37°C, except V79MZh2D6*10 which was incubated for 90 min. Samples were kept on ice for a few min and centrifuged for 10 min at 17,000 x g at 4°C. The supernatant was separated for the substrate bufuralol and its metabolite hydroxy-bufuralol by HPLC and measured by fluorimetric detection (Kronbach et al., 1987; Kronbach, 1991). Separation was achieved by isocratic means by a mobile phase consisting of 30% (v/v) acetonitrile, 40% (v/v) methanol, 30% (v/v) water, 2 mM perchloric acid, at a rate of 1 ml/min and 50°C on a Hypersil ODS C18 "*reversed phase*"-column (24 cm x 4.6 mm, particle size 5 µm; Supelco, Bellefonte, U.S.A) with C8-pre-column. Fluorescence (excitation at 252 nm, emission at 352 nm) was detected with a fluorescence HPLC monitor RF-530 (Shimadzu, Düsseldorf, Germany) and recorded with the Chromatopac C-R3A 530 (Shimadzu) with automatic integrated measurement of the area under the curve. Retention times for hydroxy-bufuralol were about 8 min and for bufuralol about 22 min. Quantification was achieved by a standardised solution of 1´-hydroxy-bufuralol in the range of 0.5 and 20 µM final concentration after incubation in homogenate of the mock-transfected cell line V79MZmockneo. The applied extinction coefficients were 16.3 $mM^{-1}cm^{-1}$ for 1´-hydroxy-bufuralol at 245.5 nm (BD Gentest, Woburn, MA, USA) and 15.1 $mM^{-1}cm^{-1}$ for bufuralol at 248 nm (Ultrafine Chemicals, London, England).

In order to measure hCYP2D6-dependent 4-hydroxylation of tamoxifen the same procedure was applied as for bufuralol-hydroxylation, except 60 min incubation for homogenate of V79MZh2D6*10 and stopping the reaction by adding 50 µl acetonitril, 2% (v/v) acetic acid. 4-hydroxy-tamoxifen was separated and detected by HPLC/ESI-MSD. Separation was achieved by an acetonitrile gradient (Fig. 1) with a flow rate of 0.5 ml/min (12th till 19th minute 0.8 ml/min) and 30°C on a C8 (2) "*reversed phase*"-column (Luna, 150 mm x 2 mm, particle size 5 µm; Phenomenex, Hösbach, Germany) with a C8-pre-column (XDB-C8, *narrow-bore column*, 2.1 mm x 12.5 mm; Zorbax HPLC Columns, Hewlett Packard, Waldbronn, Germany).

Detection of tamoxifen and its metabolite was achieved with HP Series 1100 MSD (Hewlett Packard, Waldbronn, Germany) in a *single ion monitoring modus* at m/z 344.2 (*N*-didesmethyl-tamoxifen), m/z 358.3 (*N*-desmethyl-tamoxifen), m/z 360.2 (monooxygenated metabolites of *N*-didesmethyl-tamoxifen), m/z 372.3 (tamoxifen), m/z 374.3 (monooxygenated metabolites of N-desmethyl-tamoxifen), m/z 388.3 (monooxygenated metabolites of tamoxifen) and m/z 404.3 (monooxygenated metabolites of tamoxifen-*N*-oxide).

Retention times were 2.1 min for Z-tamoxifen-3,4-epoxide, approx. 5.5 and 6.2 min for E- and Z-4-hydroxy-tamoxifen, about 7.2 and 7.9 min for E- and Z-4-hydroxy-tamoxifen-*N*-oxide, about 8.4 min for Z-tamoxifen-1,2-epoxide, 9.9 min for Z-*N*-didesmethyl-tamoxifen, 10.0 min for Z-*N*-desmethyl-tamoxifen, 10.4 min for Z-tamoxifen-*N*-oxide and about 9.7 and 10.1 min for E- and Z-tamoxifen. The peaks for E- and Z-4-hydroxy-tamoxifen, Z-tamoxifen-*N*-oxid, Z-tamoxifen-1,2-epoxide, Z-*N*-desmethyl-tamoxifen and Z-*N*-didesmethyl-tamoxifen were verified with reference compounds. Calibration was adjusted to 0.039-20 µM final concentration after incubation in homogenate of the mock transfected cell line V79MZ-mockneo.

For identification of other potential CYPs metabolically competent for tamoxifen-4-hydroxylation, homogenates of the cell lines V79MZh2E1 and V79MZh3A4-hOR and microsomal preparations of hCYP3A4 + hCYPOR and hCYP2C9*1 + hCYPOR "Supersomes™", respectively (commercially available from BDGentest, Woburn, MA, product-no. P207 and P218) have been tested with the following variation: 150 µg of V79MZh2E1 homogenate was incubated for 45 min, up to 500 µg of V79MZh3A4-hOR homogenate and up to 25 µl hCYP3A4-hOR-"Supersomes", corresponding to 50 pmol of hCYP3A4 were incubated in the presence of 100 µM $MgCl_2$ and 10 µM EDTA for 60 min and 12.5 µl hCYP2C9*1-hOR-"Supersomes", equivalent to 25 pmol of hCYP2C9*1, were incubated with 100 µM $MgCl_2$ and 10 µM EDTA for 30 min.

For inhibition studies quinidine was added to the reaction assay for bufarolol at a final concentration of 0.01 up to 2 µM starting from a stock solution of 2 mM in methanol, diluted in 0.1 M potassium phosphat buffer, pH 7.4. Control reactions were without quinidine but 0.1% methanol or with anti-hCYP2D6

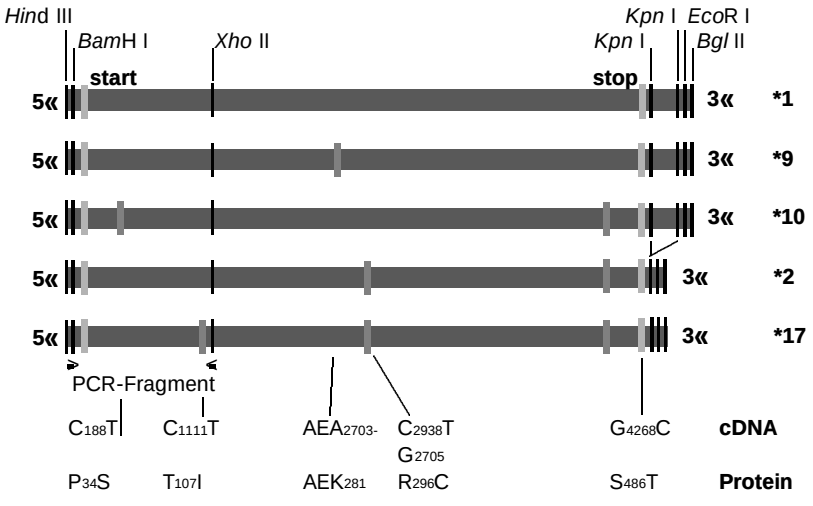

Fig. 2: The polymorphic hCYP2D6-cDNAs as inserted into expression vector pSV450h2D6. Positions of the mutations as published by Kimura et al., 1989.

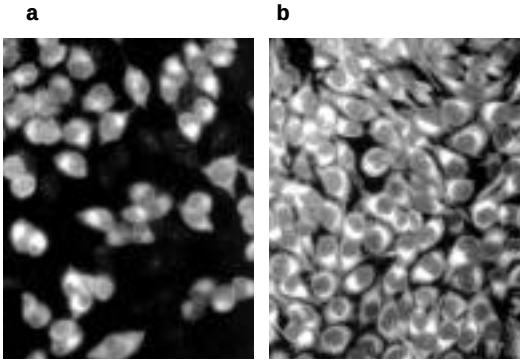

Fig. 3: *In situ* immunofluorescence with a polyclonal hCYP2D6 antibody stained with a secondary antibody conjugated to FITC. a) mixture of parental V79MZ and V79MZh2D6*1 cells. Parental cells show unspecific background fluorescence upon over exposure. b) V79MZh2D6*2 cells. Note fluorescence in the cytoplasm but not in the cell nucleus.

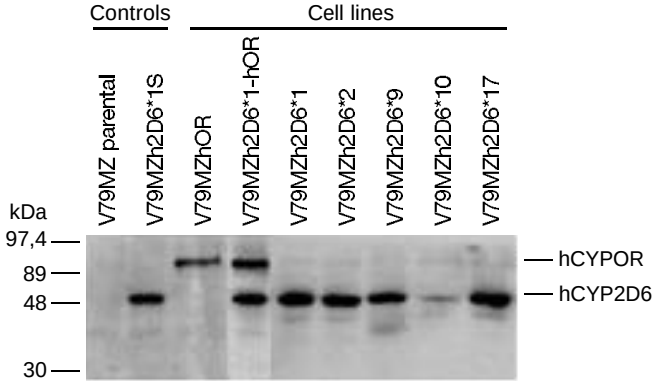

Fig. 4: Western blot of cell homogenate from all polymorphic V79MZh2D6 cell lines with and without co-expression of the human CYP reductase (hOR). A previously constructed cell line V79MZh2D6*1-S (Rauschenbach et al., 1997) served as control.

antiserum and human control serum at a final dilution of 1:100 and 1:1000.

3 Results

3.1 Recombinant expression vectors pSV450h2D6*1, *2, *9, *10, *17

The general characteristics of all recombinant pSV450h2D6 vectors are shown in Fig. 1. They are based on the prototype vector pSV450r2B1 (Doehmer et al., 1988). Of importance are the viral SV40 early promotor and the SV40 polyadenylation site, which has generally been used for the construction of all CYP recombinant V79 Chinese hamster cell lines in order to substitute for individual CYP promoters in order to be independent of induction phenomena. Thus the V79 Cell Battery™ can be standardised for constitutive expression. The individual variants *1, *2, *9, *10, and *17 are presented with their characteristic mutations in Fig. 2.

3.2 Recombinant V79 Chinese hamster cells V79MZhCYP2D6*1, *2, *9, *10, *17

Each round of transfection yielded between 30 and 50 G418-resistant colonies of which between 1 and 3 colonies maintained stable expression over a period of more than 3 months. Those cell lines showing stable integration of the recombinant cDNA were further characterised. *In situ* immunofluorescence was routinely performed to follow expression and to check cultures for homogenous expression (Fig. 3). In addition, all clones were checked by Western blotting (Fig. 4). The signal for the *10 variant was low due to intrinsic stability problems of this protein. The integrated cDNA in the V79 cells was amplified with specific primers for sequencing of each variant (Fig. 5). All variants retained their authentic sequence (Fig. 6). All integrated cDNAs were functional, expressing variant specific mRNA (Fig. 7).

For comparative enzyme kinetic studies on variants it was important to measure the CYP content per cell or per

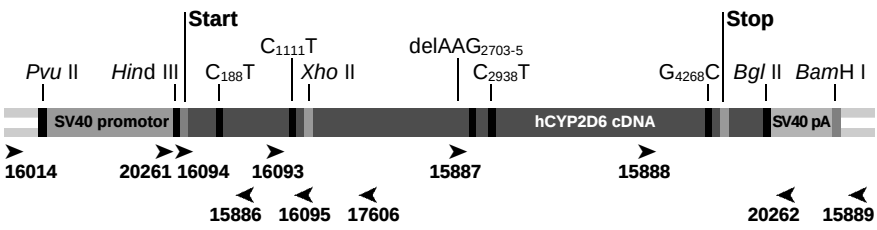

Fig. 5: Relative positions and orientation of the primers as used for all PCR reactions.

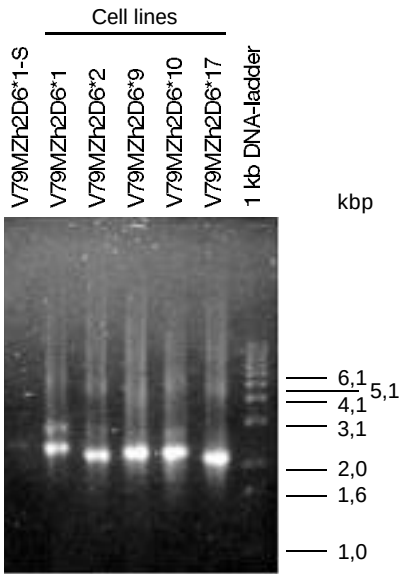

Fig. 6: PCR of the genomically integrated hCYP2D6 expression cassettes after transfection and establishment of the cell lines. V79MZh2D6*1-S (Rauschenbach et al., 1997) served as negative control. No signal obtained because of differences in the expression cassette. Primers 16014 and 15889 could not bind.

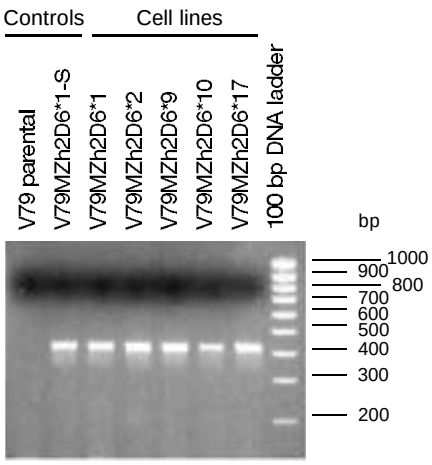

Fig. 7: Reverse transcriptase PCR on hCYP2D6-mRNA as expressed in the various polymorphic V79MZh2D6 cell lines. A 410 basepair long cDNA fragment was amplified common to all polymorphic mRNAs.

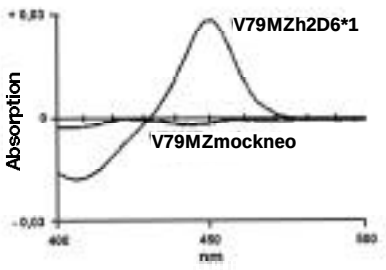

Fig. 8: CO difference spectrum of homogenate of the V79MZh2D6*1 cell line in comparison to mock transfected parental cell line V79MZmockneo showing the specific 450 nm peak for intact CYPs. Note the absence of this signal for V79MZmockneo. This is the very first general proof for the absence of CYPs in the parental V79MZ cells making the genetically engineered V79 cells a specific and unique expression system for CYPs not given for any other known cell line.

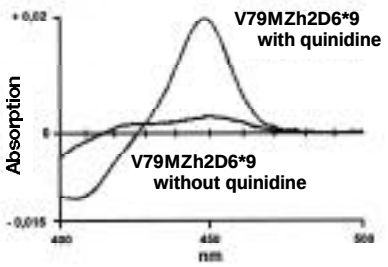

Fig. 9: CO difference spectrum of homogenate of the V79MZh2D6*9 cell line. Note the stabilising effect of quinidine when cells were cultivated in the presence of quinidine.

total protein. CO difference spectra gave 4.3 and 24.9 pmol/mg total protein for heme-containing and active CYP (Tab. 1). Except 2D6*1 (Fig. 8), all other variants also revealed a peak at 420 nm, indicating non-functional CYP between 1 and 3.8 pmol CYP per mg total protein (Tab. 1). It is worthwhile to note, that there was no detectable CYP in the parental V79MZ cells (Fig. 8). This supports earlier observations that no CYP was ever detected by Western blotting, making the recombinant V79MZ cells specific for the cDNA encoded CYP. Interestingly, quinidine had a stabilising effect which was most pronounced for 2D6*9 as shown in Fig. 9.

3.3 Variant specific metabolism of bufuralol and tamoxifen

Bufuralol is accepted as a CYP2D6 marker substrate and was applied as positive control in all other reactions. Fig. 10 shows the HPLC profile of bufuralol with a retention time of 22 min and its hydroxylated metabolite with a retention time of 8 min. In agreement with all other findings, no metabolite was detected in the homogenate of the parental V79MZ cells.

Specific activity was measured and compared (Tab. 2) with the highest activity of 162.7 pmol metabolite/mg total protein/min observed for 2D6*1 with decreasing activity for all other variants down to 3.3 pmol metabolite/mg total protein/min for 2D6*10.

Variant-specific kinetics were measured for (+)-bufuralol-1´-hydroxylation assuming monophasic Michaelis-Menten kinetics (Fig. 11). Based on these results intrinsic clearance was calculated (Tab. 3).

Tamoxifen, a selective estrogen receptor modulator, is prescribed as an anti-estrogenic drug to treat breast cancer and is known as a CYP2D6 substrate (Fig 12). Whereas bufuralol is inactivated by hydroxylation, hydroxylated tamoxifen is pharmacologically about 100fold more potent than the parent compound (Borgna and Rochefort, 1981; Furr and Jordan, 1984). Following the pro-drug concept it is important to understand variant-dependent metabolic

Tab. 1: Cytochrome P450 content in the indicated cell lines as measured by CO difference spectra without and with quinidine. (-) means not done. Values with standard deviation are based on at least 3 different and independent determinations.

	without quinidine (pmol/mg total protein)			with quinidine (pmol/mg total protein)		
Cell line	P450	P420	P450 +P420	P450	P420	P450 +P420
V79MZmockneo	0	0	0	-	-	-
V79MZhOR	0	0	0	-	-	-
V79MZh1A1	11.4±1.8	0	11.4±1.8	-	-	-
V79MZr1A1	6.8±1.9	5.6±2.7	12.4±4.7	-	-	-
V79MZm1A1	7.6±1.9	2.6±0.8	10.2±2.0	-	-	-
V79MZf1A1 (scup)	2.5±0.8	3.5±1.6	6.1±2.4	-	-	-
V79MZh2E1	5.2±0.6	0.9±1.0	6.1±0.5	-	-	-
V79MZh3A4-hOR	8.0±1.0	3.0±0.9	11.0±1.7	-	-	-
V79MZh2D6*1	24.9±3.2	0	24.9±3.2	30.9	0	30.9
V79MZh2D6*2	10.9±4.3	2.5±2.2	13.4±4.7	12.2	0	12.2
V79MZh2D6*9	5.4±2.4	1.0±0.9	6.4±1.9	35.7±13.3	0	35.7±13.3
V79MZh2D6*10	0	2.2±0.2	2.2±0.2	0	4.44	4.44
V79MZh2D6*17	4.3±0.7	3.8±0.7	8.2±1.0	9.0	1.9	10.9

Tab. 2: Bufuralol hydroxylase activity in the polymorphic V79M/h2D6 cell lines with racemic mixture of bufuralol (+/-) or enantiomers (+) or (-). Standard deviations are based on at least 3 independent determinations.

Cell line	Bufuralol-hydroxylase-activity pmol/mg total protein/min			Bufuralol-hydroxylase-turnover rate pmol/pmol hCYP2D6/min			Selectivity
	(+)	(+/-)	(-)	(+)	(+/-)	(-)	(-)/(+)
V79MZmockneo	0	0	0	-	-	-	-
V79MZh2D6*1	162.7±31.6	108.0±15.3	65.0±13.5	6.53±2.11	4.34±1.17	2.61±0.88	0.40±0.16
V79MZh2D6*2	95.2±28.6	59.7±13.9	43.8±14.7	7.11±4.63	4.46±2.60	3.27±2.24	0.46±0.29
V79MZh2D6*9	93.9±11.5	61.8±4.7	45.9±3.1	14.67±6.2	9.66±3.60	7.17±2.61	0.49±0.16
V79MZh2D6*10	3.3±0.4	2.3±0.2	1.4±0.3	1.48±0.29	1.03±0.17	0.63±0.18	0.42±0.14
V79MZh2D6*17	29.3±1.9	21.6±2.4	14.4±3.3	3.57±0.66	2.65±0.61	1.77±0.62	0.49±0.14

activation. Essentially, tamoxifen follows the same kinetics as bufuralol (Fig. 13). However, kinetic studies could not be extended to maximum turnover due to a low solubility and the need to use DMSO as solvent. It is known, that DMSO itself has an impact on CYP activity. An acceptable linear range was found for a final concentration of 2.5% DMSO (Fig. 14).

One of the major problems in CYP mediated drug metabolism is overlapping substrate specificity. Therefore, it was of interest to check for metabolic competence of other CYPs beside CYP2D6 for tamoxifen-4-hydroxylation. A significant contribution was detected for CYP2C9 (Fig. 15). Against earlier assumptions (Crewe et al., 1997), CYP3A4 does not contribute to the formation of hydroxylated tamoxifen (Fig. 15).

3.4 Inhibition studies

Drug-drug interactions are frequently observed in situations when multiple drugs are taken at the same time. The recombinant CYP V79 cells present a perfect tool to understand these interactions by inhibition studies as shown here for the impact of quinidine inhibition on bufuralol-hydroxylation (Fig. 16). Similarly, the involvement of 2C9 in the metabolism of tamoxifen explains the observed life-threatening drug-drug interactions between tamoxifen and warfarin (Lodwick et al., 1987; Rettie et al., 1992; Tennie et al., 1989).

4 Discussion

As with all other CYP recombinant V79 cells (Fig. 18) the goal of this project was the construction of CYP2D6 recombinant V79 cell lines expressing activities close to those observed in native tissue rather than achieving an overexpression of CYP as described for other CYP expression systems (e.g., Evert et al., 1997; Oscarson et al., 1997; Penman et al., 1993). Staying within a physiological range makes it easier to extrapolate from the *in vitro* to *in vivo* situation. This makes the V79 Cell Battery™ unique. CYP2D6 activities in human liver and human liver microsomes compare favourably with those detected in the recombinant V79 cells (Tab. 4).

All CYP recombinant V79 cells can be easily applied as an analytical tool to dissect complex metabolic pathways by combining and comparing different CYPs and applying synthesised metabolites of interest. This gives further insight, e.g. on competing reactions as seen for 2D6 activating tamoxifen and for 3A4 inactivating tamoxifen (Fig. 17).

Last but not least it is worthwhile to note that there is no animal model available which reflects the human CYP2D6 polymorphisms. There are only some rat strains described to be fast or slow metabolisers (Gonzalez et al., 1987; Schulz-Utermoehl et al., 1999; Vorhees et al., 1998; Yamamoto et al., 1998). It is therefore clear that V79 cells genetically engineered for stable expression of CYPs serve the first of the 3Rs of Russel and Burch, “Replacement”.

Tab. 3: Kinetic parameters of (+)-bufuralol-1´-hydroxylation for the polymorphic V79M/h2D6 cell lines. Standard deviations are based on at least 3 independent determinations.

Cell line	related to total protein			related to CYP content	
	V_{max} pmol/mg/min	K_M µM	Cl_{int} ml/mg/min	Turnover rate pmol/pmol P450/min	Cl_{int} ml/pmol P450/min
V79MZh2D6*1	170.8±15.5	13.8±1.6	12400±2580	6.9±1.5	500±170
V79MZh2D6*2	111.5±2.1	22.6±0.6	4940±220	8.3±3.1	370±150
V79MZh2D6*9	100.0±15.0	15.0±2.9	6670±1970	15.6±7.0	1040±670
V79MZh2D6*10	4.3±0.1	53.3±10.3	80±17	1.9±0.2	40±10
V79MZh2D6*17	34.3±2.8	28.2±1.3	1220±160	4.2±0.9	150±40

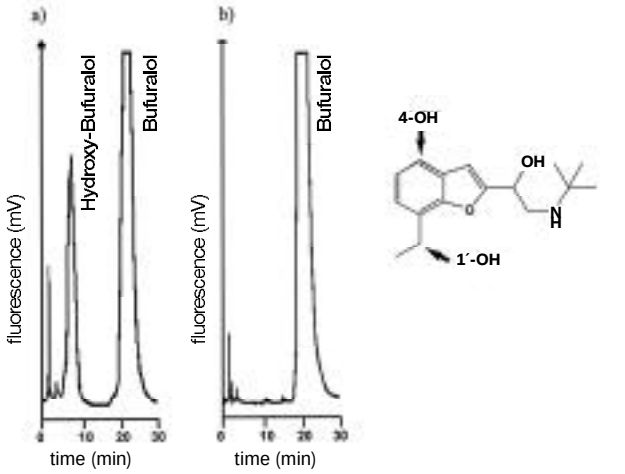

Fig. 10: HPLC profile of bufuralol incubated in homogenate of the V79MZh2D6*1 cell line (a) and of the parental V79MZmockneo cell line (b).

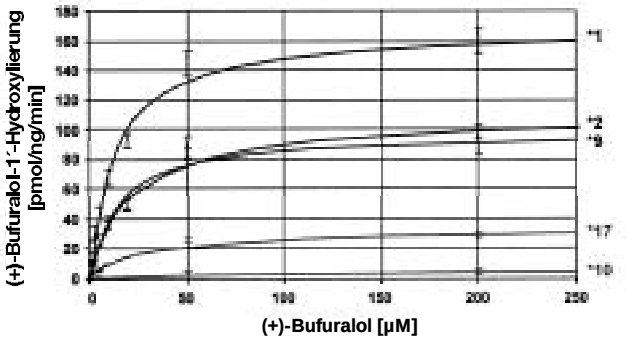

Fig. 11: Kinetics of (+)-bufuralol-1´-hydroxylation in homogenate of the polymorphic V79MZh2D6 cell lines.

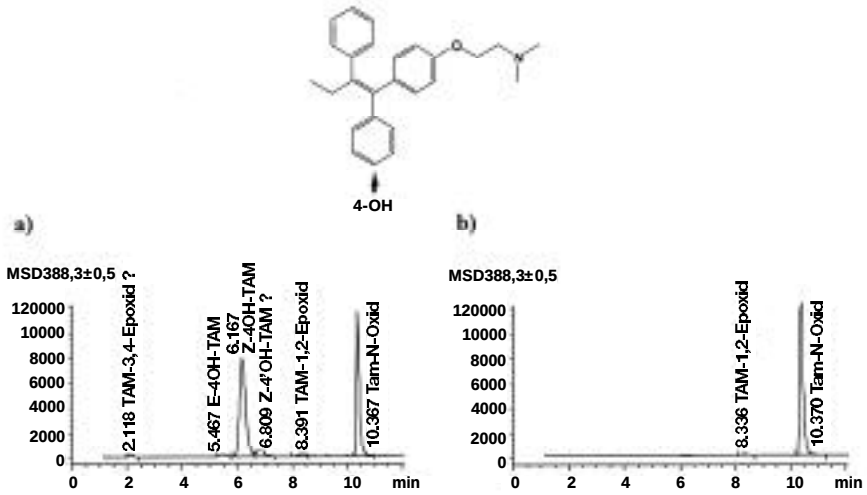

Fig. 12: HPLC/MSD profile of tamoxifen in homogenate of the V79MZh2D6*1 cell line (a) and of the parental V79MZmockneo cell line (b).

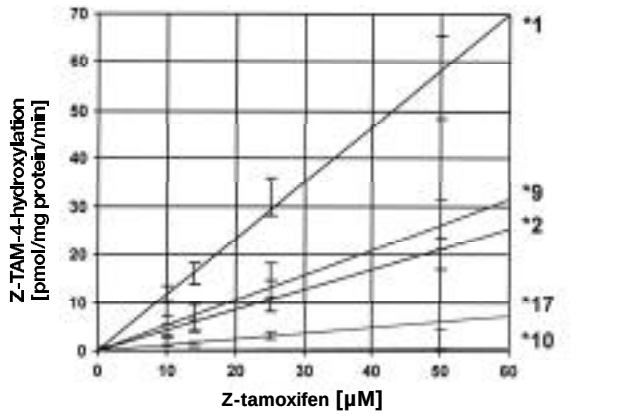

Fig. 13: Kinetics of 4-hydroxylation of tamoxifen in homogenate of the polymorphic V79MZh2D6*1 cell line in the presence of 2.5% (v/v) DMSO. Note linear increase up to 50 µM tamoxifen as determined in Fig. 12. Due to solubilisation problems concentration can not be increased to reach maximum.

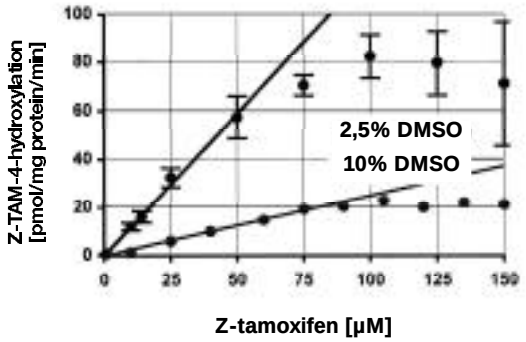

Fig. 14: Concentration dependent influence of the solvent DMSO on 4-hydroxylation of tamoxifen.

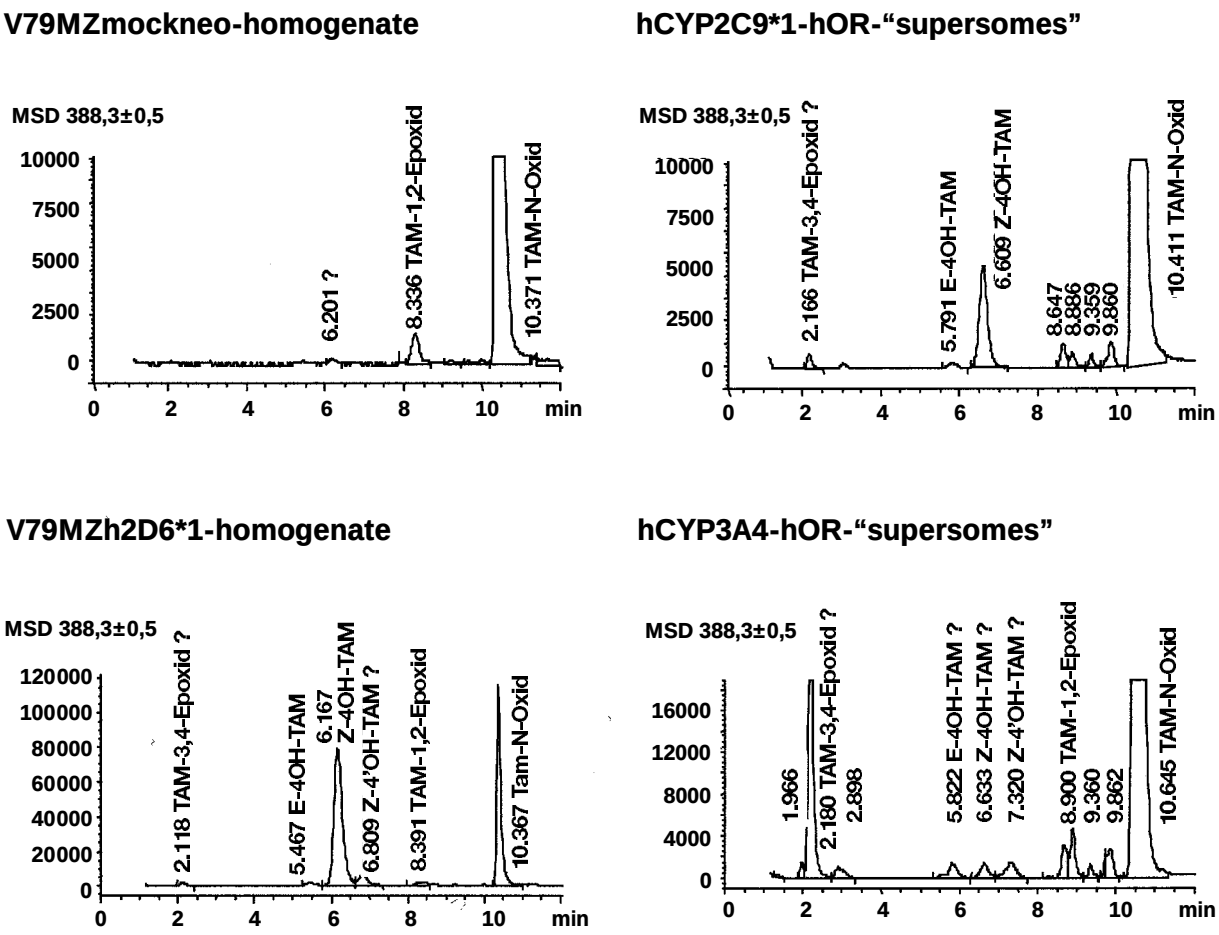

Fig. 15: HPLC/MSD profiles after incubation in various homogenates to identify metabolically competent CYPs.

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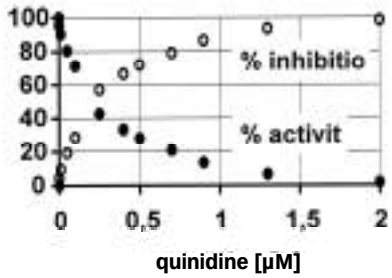

Fig. 16: Inhibition of bufuralol-hydroxylation by the hCYP2D6-specific inhibitor qunidine in the V79MZh2D6*1 cells.

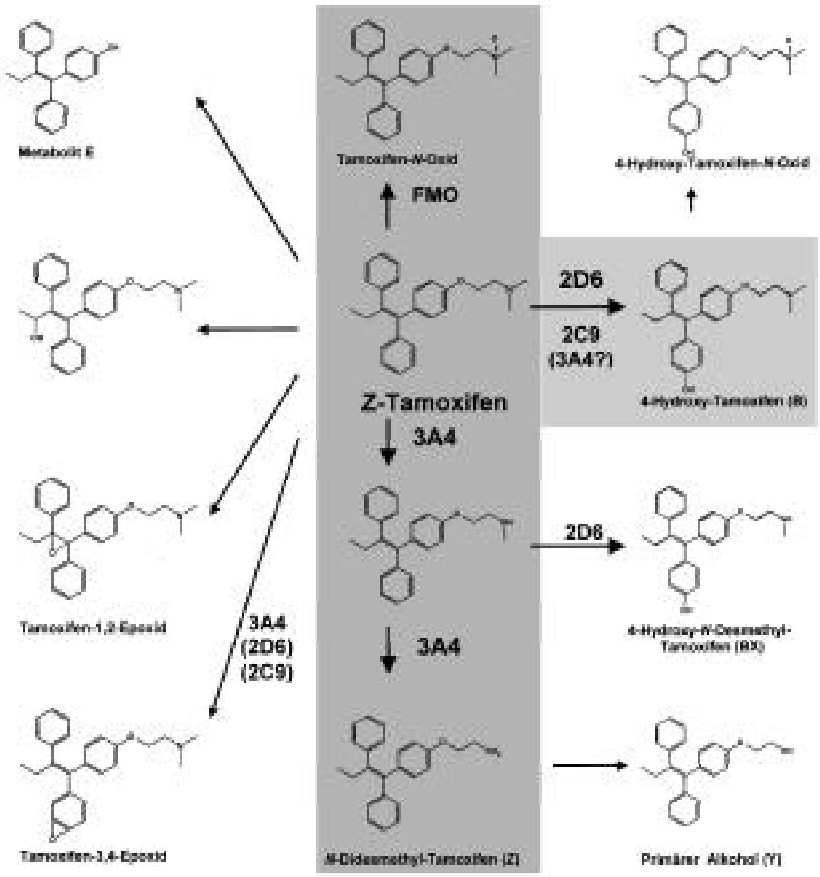

Fig. 17: Application of the V79 Cell Battery™ to unravel complete pathways, e.g. for tamoxifen.

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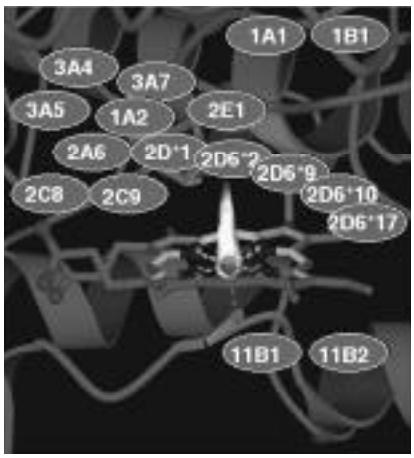

Fig. 18: Current status of the V79 Cell Battery™.

Tab. 4: Validation of V79MZ2D6*1. Comparison with values obtained from human liver microsomes for bufuralol hydroxylation.
Blanked fields means not done.

Expression system (reference)	Preparation Source of electrons	K_M µM	V_{max} (+) pmol /mg/min		Turnover rate 1/min		Selectivity
		(+)	(+)	(+/-)	(+)	(+/-)	(-)/(+)
V79MZh2D6*1	homogenate NADPH	13.8±1.6	171±16	108±15	6.9±1.5	4.3±1.2	0.40±0.16
V79MZh2D6*1 (Appel, 1999. pers. comm.)	live cells	7-8	170±10				
Human Liver[1] (Kronbach et al., 1987)	microsomes NADPH	4.7±2.2	167±43				0.56±0.17
Human Liver[2] (Dayer et al., 1987)	microsomes NADPH	17.9±6.3	199±80				0.49±0.09
Human Liver *2 (Zanger et al., 1988)	microsomes NADPH		715				0.46
Human Liver *1 (Gonzalez et al., 1988a)	microsomes NADPH		50-2400				
Human Liver *2 (Gut et al., 1986)	microsomes NADPH	47.3±8.1			0.6±0.2		0.48±0.15

*1: 2D6 genotype known

*2: Rapid metaboliser checked with sparteine and/or debrisoquine

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