EFFECT OF COLCHICINE AND ITS DERIVATIVES ON THE EXPRESSION OF SELECTED ISOFORMS OF CYTOCHROME P450 IN PRIMARY CULTURES OF HUMAN HEPATOCYTES

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The study addressed the effect of colchicine and its derivatives on the protein levels of cytochrome P450 (CYP) 1A2, 2A6, 3A4, 2C9/19, and 2E1 isoforms. Primary human hepatocyte culture was the model of choice. Levels of individual CYP isoforms were detected using immunoblotting. Colchicine caused an increase of CYP2E1 protein content, colchiceine and N-deacetylcolchiceine induced isoforms CYP2C9, 2E1 and 3A4 whereas colchicoside induced CYP2C9 and 2E1. The levels of CYP1A2 and 2A6 were unaffected by any of tested compounds. Demecolcine and 3-demethylcolchicine had no effect on any studied P450 isoform. Since colchicine is an exclusive substrate of CYP3A4 whereas it induces CYP2E1, there is a suspicion rather at protein stabilization than at gene induction concerning induction origin.

INTRODUCTION

Colchicine (COL), an alkaloid of *Colchicum autumnale*, has been used to treat gout as early as from the sixth century[1]. Endowed by antimitotic and antiinflammatory activity[2] it is currently used in therapy of familiar Mediterranean fever, Behcet's disease, cirrhosis[3, 4], progressive systemic scleroderma and amyloidosis. The great disadvantage of COL is its toxicity which hinder the common use of the substance in the therapy of diseases mentioned above. Therefore, the new colchicine derivatives were synthesized or isolated; i.e. colchiceine (EIN), N-deacetylcolchiceine (NDE), 3-demethylcolchicine (3DM), demecolcine (DEM) and colchicoside (COS); for structure see Fig. 1.

It was demonstrated recently, that the cytochrome P450 (CYP) system is involved in the metabolism of COL. The major route of COL elimination is an active biliary secretion of the parent drug and the demethylated products. Two main metabolites were identified as 3-demethylcolchicine (3DM) and 2-demethylcolchicine (2DM). The CYP3A4 isoform was found responsible for the formation of these metabolites[5]. However, possible effect of COL and its derivatives on CYP enzymes expression, a common practice in the evaluation of drug-drug interactions, was not tested as yet[6].

The aim of our work was to evaluate the effect of colchicine and its derivatives on selected isoforms of P450 (CYP1A2, CYP2A6, CYP2C9/19, CYP2E1, CYP3A4) in primary cultures of human hepatocytes on the levels of respective proteins content.

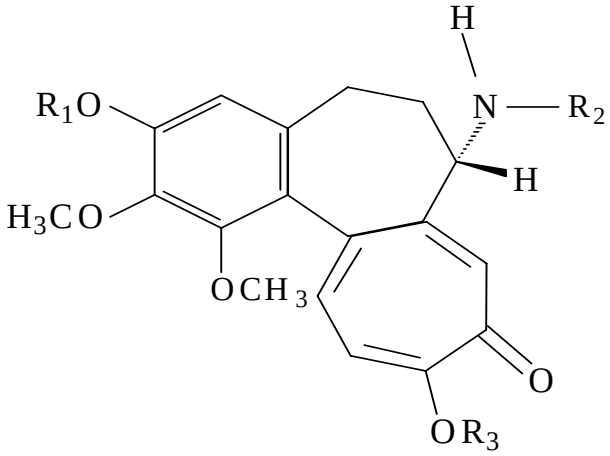

Fig. 1 Structure of colchicine and its derivatives

Compound	R_1	R_2	R_3
COLCHICINE	CH_3	$COCH_3$	CH_3
COLCHICEINE	CH_3	$COCH_3$	H
N-DEACETYLCOLCHICEINE	CH_3	H	H
3-DEMETHYLCOLCHICINE	H	$COCH_3$	CH_3
DEMECOLCINE	CH_3	CH_3	CH_3
COLCHICOSIDE	glucosyl	$COCH_3$	CH_3

MATERIALS AND METHODS

Chemicals

Colchicine and its derivatives were isolated from *Colchicum autumnale* plant. Compounds purity was checked by HPLC[7]. ISOM culture medium, rifampicin, foetal calf serum, and horseradish peroxidase conjugated secondary antibodies were purchased from Sigma (USA). Antiprotease cocktail was from Boehringer Mannheim (Germany). ECL kit including Hyperfilm photographic paper was purchased from Amersham (USA). All other chemicals and reagents were of the highest quality commercially available (Sigma).

Primary cultures of human hepatocytes

The liver samples were obtained from surgery of hepatic tumors and hepatocytes were isolated by the method of Pichard *et al*[8]. Following isolation, the cells were plated on collagen-coated culture dishes using cell density 14×10^4 cells/cm^2. Culture medium used was ISOM enriched for plating with 5% of foetal calf serum (v/v). The medium was changed for a serum-free one after 4 h stabilization. All cell culture experiments were carried out in a 5% CO_2 humidified incubator at 37 °C. Medium was exchanged after 15 h for a serum free medium containing desired concentrations of tested compounds or a corresponding volume of DMSO as a control. Medium was changed for an identical one every 24 h thereon. The total duration of induction experiments was 96 h.

Isolation of total proteins extract

Cells washed 3 times with 1.5 ml PBS buffer were scraped into 1 ml of the buffer and transferred to a 2-ml eppendorf tube kept on ice. The cell suspension was centrifuged for 5 min at 4600 × g. 400 µl lysis buffer consisting of 0.15 M NaCl, 50 mM Tris/HCl, pH 7.5, 1 mM sodium pyrophosphate, 1 mM EDTA, 0.2% Triton X-100, 2 mM dithiothreitol, 1 mM $NaVO_4$, and two tablets of antiprotease cocktail was added and the suspension vortexed. After 20 min incubation at 4 °C it was vortexed and sonicated (3 sec) twice. Supernatant resulting from another centrifugation at 15800 × g for 10 min was the total protein extract.

Isolation of microsomes

The procedure followed a general protocol for isolation of microsomal fraction from liver[9] except that the cell culture was first washed with 0.1 M phosphate buffer, pH 7.4, and then scraped off the culture dish. The initial centrifugation was followed by sonication for 45 sec.

Detection of CYP Proteins

Protein content of microsomes and total protein extracts was determined by bicinchoninic acid method[10]. SDS-PAGE gels were run on a Hoefer apparatus following the general procedure of Laemmli[11]. Protein transfer onto a nitrocellulose membrane was carried out according to Towbin *et al*[12]. Individual isoforms of P450 were detected by immunofluorescence using Amersham ECL kit. Primary antibodies, their original source stated in parentheses, used were as follows:

CYP1A2: Anti-serum from goat (Euromedex) was used in dilution 1/100. **CYP2A6:** Anti-baboon CYP2A3 raised in sheep (INSERM U128) was used in dilution 1/800 supplemented with 5% (w/v) of dried non-fat milk. **CYP2C9/19:** Antibody against denatured rat liver CYP2C6 raised in mouse (kindly provided by Drs. Waechter and Beilstein at Novartis, Basel, Switzerland) was used in dilution 1/8000 with 5% (w/v) of dried non-fat milk in the incubation buffer. **CYP2E1:** Rabbit anti-serum (a gift from Dr. Ingelman-Sundberg at the Karolinska Institute, Stockholm, Sweden) was used in dilution 1/400 with addition of 6% (w/v) dried non-fat milk. **CYP3A4:** Anti-baboon CYP3A raised in sheep (INSERM U128) was used in dilution 1/500 with the addition of 1% (w/v) dried non-fat milk.

RESULTS AND DISCUSSION

Primary cultures of human hepatocytes were treated with tested compounds in concentrations 1 and 10 µM to evaluate their effect on the expression of chosen CYP isoforms. None of the studied substances induced the CYP1A2 and 2A6 isoforms. The CYP 2C9 isoform was induced by EIN, NDE and COS in both concentrations used. The same compounds and COL induced CYP 2E1, again in both concentrations. The situation in case of CYP2C19 was ambiguous, i.e. different results were obtained for total protein extract and for microsomal fraction. The isoform CYP3A4 was induced by EIN and NDE in both concentrations. Demecolcine and 3-demethylcolchicine did not affect any of the evaluated P450 isoforms. The obtained results are attractive, because: i) As was demonstrated, for COL metabolism (O-demethylation) is exclusively responsible isoform CYP3A4. It is commonly considered, that an inducer if certain CYP isoform is usually its substrate as well. Our results suggest, that COL is not inducer of CYP3A4, whereas it induces CYP2E1. ii) Treatment of cells by COL, EIN, NDE and COS resulted in an increase of CYP2E1 protein level. Currently, only a few inducers of this isoform is known, including ethanol or acetone.

At this point it should be regarded, that in terms of induction is meant the increase in respective CYP protein content in comparison with untreated cells. The pattern of induction can be basically either intrinsic induction of corresponding gene transcription or stabilisation of resulting protein by various mechanisms. To distinguish the induction mechanism, the CYP mRNAs should be analysed.

Since there were not observed marked differences between either used concentrations of tested compounds, the repetition experiment (culture FT151) was ran using just 10 µM concentration of studied substances, with exception of COL and COS (used in 1 µM) which ex-

erted high toxicity at 10 μM observed microscopically as burst cells.

Western blotts of CYP proteins are shown in Fig. 2. For each isoform and substance were calculated the induction ratios (Table 1).

Table 1. Induction ratios of CYP1A2, CYP2C9, CYP2E1 and CYP3A4.

Compound	(μM)	CYP 1A2	2C9	2 E1	3A4
COLCHICINE	1	0.71	0.96	1.81	0.78
	10	0.49	0.66	1.49	0.45
COLCHICEINE	1	0.63	1.35	2.07	1.69
	10	0.99	1.37	2.61	1.74
N-DEACETYLCOLCHICEINE	1	0.87	1.65	2.18	2.52
	10	0.98	1.25	2.70	1.77
3-DEMETHYLCOLCHICINE	1	0.41	0.87	0.77	0.01
	10	0.57	0.76	0.75	0.52
DEMECOLCINE	1	0.06	0.83	0.28	0.05
	10	0.56	0.58	0.60	0.45
COLCHICOSIDE	1	0.65	2.28	2.70	1.04
	10	1.01	3.73	1.45	0.85
RIFAMPICIN	25	-	1.70	-	3.56
DIOXIN	0.01	3.41	-	-	-

Values are expressed as mean from two experiments; SD was less than 0.1 (for CYP1A2, CYP3A4), resp. 0.2 (for CYP2C9, CYP2E1). IR (induction ratios) were calculated from formula: IR = band surface of tested compound/band surface of corresponding control. For untreated cells IR = 1.00.

CONCLUSION

Studied colchicinoids had no effect on the CYP1A2 and CYP2A6 isoforms. The isoform CYP2C9 was induced by EIN, NDE and COS, isoform CYP2E1 by COL, EIN, NDE and COS and isoform CYP3A4 by EIN and NDE. Ambiguous results were obtained for CYP2C19 isoform. COL is an inducer of CYP2E1 isoform (but not CYP3A4), even though it is the substrate of CYP3A4. No effect on induction of studied P450 isoforms was observed at demecolcine and 3-demethylcolchicine. The mechanism of inductions is not yet clear and will be elucidated by the experiments with CYP mRNAs and by activity studies. The most interesting seems to be CYP2E1 induction.

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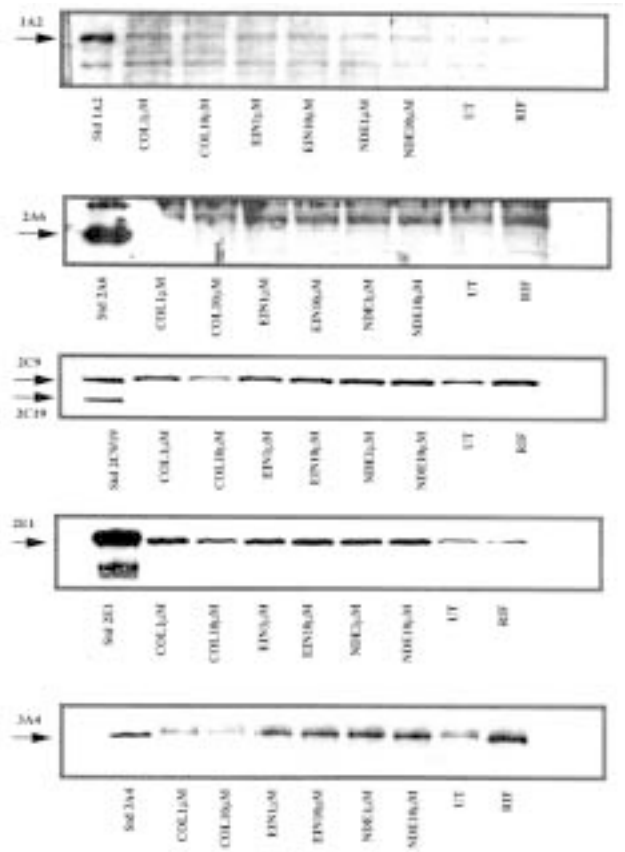

Fig. 2a Western blotts of CYP1A2, CYP2A6, CYP2C9, CYP2C19, CYP2E1 and CYP3A4.
Abbreviations used are described in text. The total treatment duration was 96 h with medium changing every 24 h.

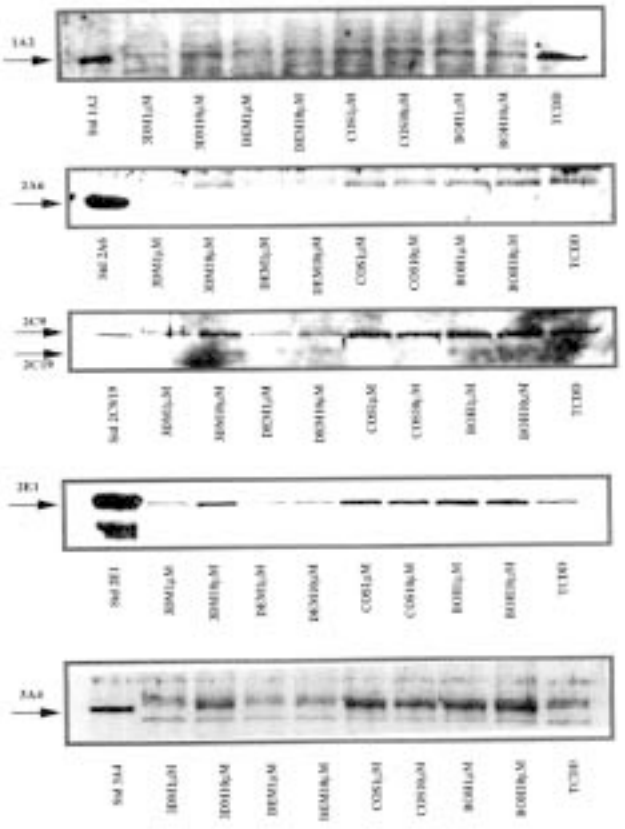

Fig. 2b. Western blotts of CYP1A2, CYP2A6, CYP2C9, CYP2C19, CYP2E1 and CYP3A4.
Abbreviations used are described in text. The total treatment duration was 96 h with medium changing every 24 h.

REFERENCES

1. Walace, S. L. (1974) Colchicine. Semin. Arthritis Rheum. *3*, 369–381.
2. Brossi, A., Yeh, H. J., Chrzanowska, M., Wolff, J., Hamel, E., Lin, C. M., Quin, F., Suffness, M., Silverton, J. (1988) Colchicine and its analogues: recent findings. Med. Res. Rev. *8*, 77–94.
3. Kaplan, M. M., Schmid, C., Provenzale, D., Sharma, A., Dickstein, G. and McKusick, A. (1999) A prospective trial of colchicine and methotrexate in the treatment of primary biliary cirrhosis. Gastroenterology *117*, 1173–1180.
4. Ben-Chetrit, E., Levy, M. (1998) Colchicine: 1998 update. Semin. Arthritis Rheum. *28*, 48–59.
5. Tateishi, T., Soucek, P., Caraco, Y., Guengerich, F. P., Wood, A. J. J. (1997) Colchicine biotransformation by human liver microsomes. Biochem. Pharmacol. *53*, 111–116.
6. Li, A. P., Maurel, P., Gomez-Lechon, M. J., Cheng, L. C., Jurima-Romet, M. (1997) Preclinical evaluation of drug-drug interaction potential: present status of the application of primary human hepatocytes in the evaluation of cytochrome P450 induction. Chem.-Biol. Interact. *107*, 5–16.
7. Husek, A., Sutlupinar, N., Sedmera, P., Voegelein, F., Válka, I., Šimánek, V. (1990) Alkaloids and Phenolics of *Colchicum turcicum*. Phytochemistry *29*, 3058–3060.
8. Pichard, L., Fabre, I., Fabre, G., Domerque, J., Aubert, B. S., Mourad, G., Maurel, P. (1990) Cyclosporin A drug interactions. Screening for inducers and inhibitors of cytochrome P450 (cyclosporin A oxidase) in primary cultures of human hepatocytes and in liver microsomes. Drug Metab. Dispos. *18*, 595–606.
9. Schenkman, J. B., Jansson, I. (1998) Isolation and purification of constitutive forms of microsomal cytochrome P450. In: Methods in Molecular Biology, Vol. *107*: Cytochrome P450 Protocols. (Eds. Phillips IR and Shephard EA), 55–67, Humana Press Inc., Totowa, New Jersey.
10. Stoscheck, C. M. (1990) Quantitation of protein. In: Methods in Enzymology, Vol *182* (Ed. Deutscher MP), 50–69, Academic Press, New York.
11. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) *227*, 680–685.
12. Towbin, H., Staehelin, T., Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. U.S.A. *76*, 4350–4354.