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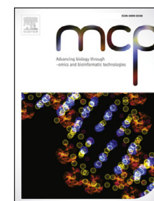

Comparison of in vitro methods for carboxylesterase activity determination in immortalized cells representative of the intestine, liver and kidney

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ABSTRACT

Herein we compare the fluorimetric determination of total and specific carboxylesterase activity in immortalized human derived living cells and in cell lysates. The cell lines chosen are representative of metabolism occurring in the intestine (Caco-2 and HT-29), kidney (HEK-293T) and liver (Hep G2). Caco-2 and HT-29, as cells prone to differentiation, were tested along the differentiation time. For evaluation of both methods when distinguishing activity of different carboxylesterases, HEK-293T transfected with the human carboxylestarase-2 (hCES2) were also tested.

Application to Caco-2 or HT-29 cells demonstrated higher activity detected in cell lysates than in cell monolayers. The difference is most striking when comparing the methods at different stages of Caco-2 and HT-29 cell maturation, highlighting substrate accessibility as a limiting step in the *in vivo* hydrolysis rates (possibly limited by plasma and Endoplasmic Reticulum membrane permeability) with increasing relevance as the cells differentiate. Application to Hep G2 or to hCES2 transfected and non-transfected HEK-293T cells, demonstrated a tendency for higher sensitivity in living cell suspensions than that obtained with the cell lysates which indicates the importance of cell environment in the maintenance of enzyme activity. However, quantification of hCES2 activity relative to total esterase, or to total carboxylesterase activity, was not significantly different in any case.

The results herein presented help to clarify which method is best suited for evaluation of carboxylesterase activity in vitro depending on the final goal of the study.

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1. Introduction

Carboxylesterases (CESs) (EC 3.1.1.1) are phase-I drug metabolising enzymes of the serine hydrolase family [21], which catalyse the conversion of carboxylic esters to the corresponding alcohols and carboxylic acids, such as pyrethroid pesticides, drugs of abuse (cocaine, heroin), prodrugs (temocapril, cilaprazil, quinapril) or anti-tumour drugs (irinotecan and capecitabin). Human CESs (hCESs) are classified in five families, hCES1 to 5, including carboxylesterase 1 (hCES1) and carboxylesterase 2 (hCES2) which are the predominant CES in liver and small intestine, respectively [8].

CESs can be found in the plasma of several species such as mouse, rat, rabbit, horse and cat but human CESs are exclusively detected intracellularly due to the presence of a C-terminal retention sequence, through which the enzymes become anchored inside the Endoplasmic Reticulum (ER) [7,15,25].

Different methods have been developed for studying the activity of hCESs in cell lysates [13] as well as in living cells [17]. Hydrolysis by hCES is, in fact, most commonly tested in vitro using tissue homogenates, microsomes or cell lysates [21]. However, cell lysates or sub-cellular fractions such as microsomes do not mimic the endogenous physiological environment [12] and may contribute to determinations that do not truly represent *in vivo* response, as reported for both UGTs and cytochrome P450 [22] and [23].

The extraction of an enzyme from its cellular environment, as

Abbreviations: BNPP, bis-*p*-nitrophenyl phosphate; Caco-2, human colon adenocarcinoma cell line (DSMZ ACC 169); CES, carboxylesterase; HBSS, hanks balanced salt solution; hCES, human carboxylesterase; HEK-293T, human embryonic kidney cells (CRL-11268, ATCC); Hep G2, hepatocellular derived carcinoma cell line (ATCC HB-8065™); HT-29, human colon adenocarcinoma cell line (ATCC HTB-38™); 4-MUB, 4-methylumbelliferone; 4-MUBA, 4-methylumbelliferyl acetate.

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occurs during cell lysis, may impact factors such as its tertiary structure, aggregation state and the interactions with other proteins or its stability through the release of proteases that would otherwise be compartmentalized [25,28]. Furthermore, the ability of a substrate or inhibitor to reach an intracellular enzymatic target, may also affect the observed activity *in vivo* in comparison with that observed in cell fractions or lysates [16].

Increased attention has been devoted to the analysis of proteins in whole-living cells, resorting to different tools such as fluorescent probes (allowing the analysis of cytoplasmic membrane anchored enzymes such as alkaline phosphatase [2] or protein–protein interactions [6]) and molecular biology techniques (enabling quantification of the activity of a cytoplasmic enzyme by coupling it to a reporter enzyme such as luciferase [1]). Recent advances have been made also in the quantification of ER localized enzyme activities, using specific CES fluorescent probes [9,27] that have been applied for live imaging [5].

The presence of CESs has been confirmed in immortalized cell lines that are commonly used as models for the study of drug metabolism and for recombinant protein expression. Most withstanding, Caco-2, a human colon adenocarcinoma isolated cell line [3] that spontaneously differentiates in culture presenting characteristics of human enterocytes [20] expresses both hCES1 and hCES2 although the relative expression levels are different from those found in the enterocytes [10]. *hCES2* gene has also been reported to be present in another intestinal derived cell line (HT-29) at approximately half the level of hCES1 but no conversion of the hCES2 substrate irinotecan has been observed, indicating the absence of active hCES2 in these cells [19]. Both hCES1 and hCES2 are present in Hep G2, a liver derived cell line used for metabolism studies [18]. General carboxylesterase activity is present in the kidney derived HEK-293T cell line. Although such activity is not due to hCES2, the protein can successfully be recombinantly expressed in this cell line [14].

The goal of the work herein presented was to compare two methods (celllysate analysis and whole living cell analysis), for determining the hydrolytic activity of hCESs using as test systems, these human immortalized cell lines.

2. Material and methods

2.1. Reagents

4-Methylumbelliferyl acetate (4-MUBA, ≥ 98%), 4-methylumbelliferone (4-MUB, ≥ 98%), potassium chloride (KCl, >99%), potassium di-hydrogen phosphate (KH_2PO_4, >99%), sodium chloride (NaCl, >99.5%), ethylenediaminetetraacetic acid (EDTA, > 98.5%), triton X-100, loperamide hydrochloride (≥99.8%), bis-*p*-nitrophenyl phosphate (BNPP, > 99%), trizma hydrochloride (>99%), trizma base (>99.9%), dimethyl sulfoxide (DMSO, ≥ 99.9%) and procaine hydrochloride (≥97%) were purchased from Sigma–Aldrich (St. Louis, U.S.A.).

2.2. Caco-2 cells culturing and preparation of lysates

Human colon adenocarcinoma (Caco-2) cells, (DSMZ ACC 169; Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany), were cultured in Roswell Park Memorial Institute medium (RPMI) 1640 media (Life Technologies; Grand Island, U.S.A.) supplemented with 10% (*v/v*) foetal bovine serum (FBS; Life Technologies), 2 mM L-glutamine (Life Technologies) and 1% of 5000 U mL^{-1} penicillin-streptomycin solution (Life Technologies) at 37 °C and 5% (*v/v*) CO_2. Cells were routinely sub cultured once a week, before reaching confluence, using 0.25% (*w/v*) Trypsin-EDTA (Life Technologies). Determination of cell concentration and viability was performed with a Fuchs–Rosenthal counting chamber (Marienfeld, Lauda-Königshofen, Germany) using 0.4% (*w/v*) trypan blue (Gibco; Carlsbad, U.S.A.) staining. Media replacement was performed every other day.

For activity assays, Caco-2 cells were seeded in 96 well plates at passage 28 with an inoculum of 1.04×10^4 cells cm^{-2} and cultured in 100 μL of RPMI. The medium was replaced with the same volume of fresh medium three times a week for 21 days. For preparation of cell lysates, cells from three wells were lysed with 50 mM Tris–HCl, 2% triton X-100, 5 mM EDTA and 0.25 mM NaCl, after determination of cell number and viability using trypan blue as detailed above. Confirmation of the full extent of cell lysis was performed using a counting chamber as previously described. Following cell lysis, centrifugation at 10,000 rpm was performed for 10 min at 4 °C.

2.3. HT-29 cells culturing

HT-29 human colon adenocarcinoma cell line (ATCC HTB-38™) was cultured in RPMI 1640 high glucose medium (Life Technologies) supplemented with 10% (*v/v*) FBS and 2 mM L-glutamine, at 37 °C and 5% (*v/v*) CO_2. Cells were routinely sub cultured once a week before reaching confluence, using 0.25% (*w/v*) Trypsin-EDTA. Determination of cell concentration and viability was performed as described above.

For activity assays, culture medium was changed to RPMI 1640 medium without glucose (Life Technologies), supplemented with 10% (*v/v*) FBS and 5 mM galactose (Life Technologies) before seeding. Cells were then seeded in 96-well tissue culture plates at passage 32, with a seeding inoculum of 3.16×10^4 cell cm^{-2}. Media replacement was performed three times a week during 21 days. Cell lysates were prepared as described for Caco-2 cells above.

2.4. Hep G2 cells culturing and preparation of cell lysates

The hepatocellular derived carcinoma Hep G2 cell line (ATCC HB-8065™) was cultured in Minimum Essential Medium Eagle (MEM; Life Technologies) supplemented with 10% (*v/v*) FBS, 2 mM of glutamine, 1 mM sodium pyruvate (Life Technologies) and 0.1 mM of non-essential amino acids solution (NEAA; Life Technologies), at 37 °C and 5% (*v/v*) CO_2. Cells were routinely sub cultured once a week. Determination of cell concentration and viability was performed as described above.

For activity assays, Hep G2 cells were seeded in 96-well tissue culture plates at passage 121 with a seeding inoculum of 1.88×10^4 cell cm^{-2}, and tested 24 h after seeding. For preparation of extracts, cells from three wells were lysed using Mammalian Protein Extraction Reagent (M-PER) from Pierce Biotechnology (Rockford, U.S.A.) after determination of cell number and viability using trypan blue as detailed above. Following cell lysis, centrifugation at 10,000 rpm was performed for 10 min at 4 °C.

2.5. HEK-293T cells culturing

Previously developed suspension-adapted human embryonic kidney cells (HEK-293T; CRL-11268, ATCC) expressing active hCES2 were used [14]. Briefly, cells were transfected with 10 μg mL^{-1} of pCI-neo-CES2 vector and incubated at 37 °C and 8% (*v/v*) CO_2 in air, using orbital agitation (130 rpm) for 48 h post-transfection (hpt). Cells were routinely maintained in suspension cultures using FreeStyle 293 Expression Medium (Gibco; Grand Island, U.S.A.), at 37 °C and 8% (*v/v*) CO_2 in air, using orbital agitation (130 rpm) and were routinely sub cultured twice a week, using an inoculum of 0.5×10^6 cell mL^{-1}. For activity assays on living cells, HEK-293T and HEK-293T hCES2 transfected cells were transferred to 96-well plates at 17×10^3 cell $well^{-1}$, and assayed immediately. For

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