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Improving the *ex vivo* stability of drug ester compounds in rat and dog serum: Inhibition of the specific esterases and implications on their identity

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ABSTRACT

In drug development, it has been noticed that some drug compounds, especially esters, are unstable in serum samples *ex vivo*. This can lead to a substantial underestimation of the actual drug concentration.

The rat and the dog, representing a rodent and non-rodent species, respectively, are widely used in preclinical studies. We studied the degradation of three structurally different drug esters in rat and dog serum. Moreover, the efficiency of selected enzyme inhibitors to prevent these degradations was investigated. Furthermore, we found indications of the identity of the drug-specific esterases by means of their inhibitor sensitivity as well as by protein purification and identification. The studied drugs were sagopilone, drospirenone, and methylprednisolone aceponate (MPA) all of which are used in (pre-)clinical drug development.

The sagopilone-cleaving esterases in rat serum were inhibited by serine hydrolase inhibitors. We partly purified these esterases resulting in an activity yield of 5% and a purification factor of 472. Using matrix-assisted laser desorption ionization (MALDI)–time of flight (TOF)–mass spectrometry (MS), the rat carboxylesterase isoenzyme ES-1 was identified in these fractions, thus pointing to its involvement in sagopilone cleavage. Drospirenone cleavage in rat serum was effected by butyrylcholinesterase (BChE) and paraoxonase 1 (PON1) as we deduced from the high efficacy of certain serine hydrolase and metallohydrolase inhibitors, respectively. Likewise, some inhibition characteristics implied that MPA was cleaved in rat serum by BChE and serine proteases. Partial purification of the MPA-specific esterases resulted in activity yields of 1–2%, exhibiting up to 10,000-fold purification.

In dog serum, we found that sagopilone was not degraded which was in contrast to MPA and drospirenone. MPA degradation was mainly prevented by serine hydrolase inhibitors. We used a three-step purification to isolate the esterases cleaving MPA. This procedure resulted in an activity yield of 12% and 645-fold purification. By protein identification using liquid chromatography (LC)–electrospray ionization (ESI)–MS, we identified α_2-macroglobulin (α_2M) in the active fractions. We therefore assumed that serine hydrolases, probably butyrylcholinesterase, known to form esteratically active complexes with α_2M, were responsible for MPA cleavage. In contrast, PON1 was assumed to be involved in drospirenone cleavage due to the high efficiency of metallohydrolase inhibitors. This indication was supported by the presence of PON1 in drospirenone-cleaving fractions as we found by affinity chromatography and Western immunoblotting for isolation and detection of PON1, respectively.

The identity of the assumed cleaving enzymes remains, however, to be further studied. The inhibitors we found can serve as a tool for stabilizing drug ester compounds in biological samples *ex vivo*.

Abbreviations: α_2M, α_2-macroglobulin; AC, affinity chromatography; AChE, acetylcholinesterase; ACN, acetonitrile; albumin-AC, affinity chromatography for removal of albumin; APMSF, 4-amidinophenylmethanesulfonyl fluoride hydrochloride; BCA, bicinchoninic acid; BChE, butyrylcholinesterase; BNPP, bis(*p*-nitrophenyl) phosphate sodium salt; $CaCl_2$, calcium dichloride dihydrate; cDNA, complementary deoxyribonucleic acid; CE, carboxylesterase; ChE, cholinesterase; EDTA, ethylenediamine tetraacetic acid disodium salt dihydrate; EGTA, ethyleneglycol-bis(2-aminoethylether)-tetraacetic acid; IEC, anion-exchange chromatography; irinotecan, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin, also referred to as CPT-11; LC-ESI-MS, liquid chromatography–electrospray ionization–mass spectrometry; MALDI-TOF-MS, matrix-assisted laser desorption ionization–time of flight–mass spectrometry; MPA, methylprednisolone aceponate; NaCl, sodium chloride; NaF, sodium fluoride; NCBI, National Center for Biotechnology Information (Bethesda, USA); NPA, 4-nitrophenyl acetate; PAF-AH, platelet-activating factor acetylhydrolase; PCR, polymerase chain reaction; Pefabloc® SC, 4-(2-aminoethyl-)benzenesulfonyl fluoride hydrochloride; PON, paraoxonase; PON-AC, affinity chromatography for isolation of PON1-containing fractions; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SEC, size-exclusion chromatography; TBA, tetrabutylammonium bromide; TBS, Tris buffered saline; TFA, trifluoroacetic acid; TLCK, *N*-α-tosyl-L-lysine chloromethylketone hydrochloride; Tris, tris(hydroxymethyl)aminomethane; Tris–HCl, tris(hydroxymethyl)aminomethane hydrochloride; UV–vis, ultraviolet–visible spectroscopy.

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1. Introduction

The stability of drugs in biological samples plays a crucial role in drug development since preclinical and clinical studies require correct drug quantitation with bioanalytical methods. The drug stability mainly depends on the chemical properties of the drug (e.g. the presence of certain functional groups), on the biological properties of the sample matrix (e.g. enzyme activities) as well as on the storage conditions of the drug-containing samples (e.g. temperature conditions). The degradation of drugs after sampling will certainly result in a drug concentration below the true concentration on the time point of sampling. Therefore, authorities responsible for drug marketing approval have published some guidelines for drug development studies in animals and humans in order to examine and to ensure sample stability from sampling to the actual sample analysis [1–4].

The instability of drugs in biological samples was clearly exemplified by drug ester compounds in blood, plasma or serum samples [5,6]. The degradation of drug esters in these matrices was ascribed to esterase activities [7,8]. Esterases belong to the class of enzymes splitting an ester hydrolytically into an acid and an alcohol (hydrolases; EC 3.1) [9,10]. Among others, the following esterases are present in animal and human plasma and serum: acetylcholinesterase (AChE; EC 3.1.1.7), butyrylcholinesterase (BChE; EC 3.1.1.8), carboxylesterase (CE; EC 3.1.1.1), and paraoxonase (PON; EC 3.1.8.1; formerly known as arylesterase) [7,9,11,12]. AChE, BChE and CE are serine hydrolases, i.e. they possess a catalytically active serine residue in their active sites as part of the catalytic triad [9]. In contrast, a catalytic dyad of two histidine residues was found in PON and assumed to be responsible for the esterase and lactonase activities of PON [13]. Furthermore, calcium ions play an important role in the conformational stability and catalytic activity of PON [13], thereby emphasizing the classification of PON as a calcium-dependent metallohydrolase [9]. BChE, CE and PON are considered to be the most relevant esterases in plasma or serum in terms of drug ester cleavage since they exhibit relatively high concentrations in these matrices, on the one hand, and relatively low substrate specificities (as compared to AChE), on the other hand [7,14]. However, there were some drugs which were mainly hydrolyzed by serum albumin [7]. In some cases, the used albumin preparations were contaminated with BChE, thus associating the esterase activity with BChE rather than with albumin [9].

In drug development, one has become aware of the fact that certain drug ester compounds underwent rapid degradation, especially, in rat serum *ex vivo*. The rat is a typical and widely used rodent species in preclinical studies whereas the dog represents an often employed non-rodent species. When compared to rat serum, the esterase activities of dog and human serum are similar to a great extent. This was demonstrated, on the one hand, quantitatively by incubation studies and, on the other hand, qualitatively by gel electrophoresis studies [15,16]. The esterase activities in rat serum have mostly been reported to be considerably higher than in dog or human serum concerning certain ester compounds [17–22]. In contrast, there are some drug esters which were described to have similar or higher stability in rat serum or plasma compared with the corresponding matrices of dogs or humans [23–26]. Rat serum was found to exhibit a broad CE band near the albumin band in gel electrophoresis [15]. This intensive CE band is, however, not present in dogs and humans [15]. Accordingly, the CE activity of rat serum was reported to be distinctly higher than in dogs or humans, leading to the conclusion that dog and human serum do not contain CE in relevant concentrations [7,27–30]. Rat serum or plasma have, on the other hand, a 3–9-fold lower cholinesterase (ChE) activity than the corresponding dog or human matrix [31,32]. It can thus be assumed that CE substrates may be cleaved faster in rat serum than in dog or human serum. This is in contrast to the hydrolysis of BChE substrates. For instance, irinotecan (7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin, also referred to as CPT-11) is a CE substrate which is indeed cleaved more rapidly in rat serum than in human serum [19,33]. Procaine is well known as a BChE substrate which is rapidly degraded in dog and human serum, but stable in rat serum [7,25].

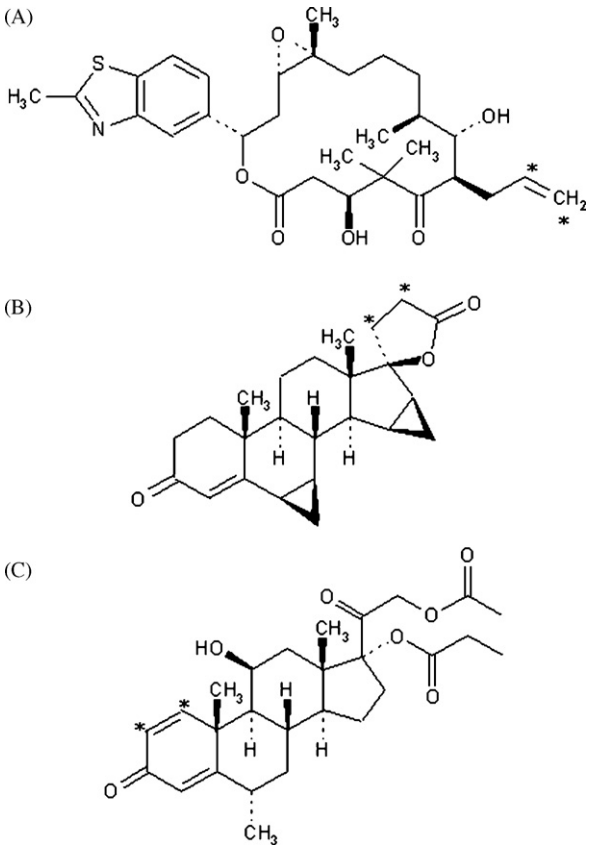

Fig. 1. Structural formula of (A) sagopilone, (B) drospirenone, and (C) methylprednisolone aceponate (MPA). An asterisk marks the position of a ^{3}H-label.

The aim of the present study was to investigate and to improve, where appropriate, the stability of the three structurally different drug esters sagopilone, drospirenone and methylprednisolone aceponate (MPA) in rat and dog serum. Esterase inhibitors were often used successfully for improving the stability of drug ester compounds in animal and human blood, plasma or serum [5,6,34]. In our study, we investigated the efficiency of certain esterase inhibitors to protect the studied drug esters from hydrolysis in rat and dog serum. Furthermore, the different inhibitor sensitivity gave implications on the identity of the responsible esterases. In addition, we isolated the esteratic fractions from native serum and used them for protein identification in order to explain the different inhibitor efficiency towards the drug-cleaving esterases.

In the present study, we investigated the esteratic cleavage of three structurally different drug esters (sagopilone, drospirenone, and methylprednisolone aceponate [MPA]) in rat serum *in vitro*. These drugs have long been or are currently studied in preclinical and clinical drug development, especially at Bayer Schering Pharma AG. Improving their stability in biological matrices is of utmost importance for on-going and future pharmacokinetic studies with these and structurally related compounds. We have chosen these three drugs since they represent different classes of drug esters: Sagopilone (also referred to as ZK-EPO) is a 16-membered *macrolactone* (an epothilone derivative) and presently under clinical investigation as an anti-tumour drug (Fig. 1A) [35]. Drospirenone can be classified as a *steroid lactone* which structurally resembles

spironolactone, both carrying a γ-butyrolactone ring at the spiro-carbon atom in position 17 of the steroid structure (Fig. 1B). It is a well-established gestagen used for oral hormonal contraception and postmenopausal hormone replacement therapy [36]. In contrast, MPA is a *corticosteroid with two open-chain ester groups* at position 17α and 21 (Fig. 1C). It is therapeutically used for topical treatment of various forms of dermal eczema as well as of sunburn, psoriasis and atopical dermatitis [37]. The three investigated compounds were tritium-(^{3}H-)labeled for radiochemical HPLC analysis.

In the present study, we found efficient esterase inhibitors which can subsequently be used for stabilization of the three investigated drugs as well as structurally related ester drugs in serum samples during preclinical and clinical studies *ex vivo*.

2. Materials and methods

2.1. Chemicals

All chemicals were of analytical reagent grade unless otherwise stated.

The ^{3}H-labeled drug ester compounds sagopilone ((1*S*,3*S*,7*S*,10*R*,11*S*,12*S*,16*R*)-7,11-dihydroxy-8,8,12,16-tetramethyl-3-(2-methyl-1,3-benzothiazol-5-yl)-10-([2,3-$^3H_2$]prop-2-enyl)-4,17-dioxabicyclo[14.1.0]heptadecane-5,9-dione; Fig. 1A), drospirenone (6β,7β,15β,16β-dimethylene-3-oxo-17α-pregna-4-ene-21,17-[20,21-$^3H_2$]carbolactone; Fig. 1B), and MPA (21-acetoxy-11β-hydroxy-6α-methyl-17-propionyloxy-1,4-[1,2-$^3H_2$]pregnadiene-3,20-dione; Fig. 1C) were synthesized by Bayer Schering Pharma AG (Berlin, Germany). 4-Nitrophenyl acetate (NPA; 99%) and tris(hydroxymethyl)aminomethane hydrochloride (Tris–HCl; 99%) were purchased from Alfa-Aesar (Karlsruhe, Germany). Acetonitrile (ACN; gradient grade), calcium dichloride dihydrate ($CaCl_2$), glacial acetic acid (acetic acid, 100%), ethanol (100%), glycerine (87%), water for HPLC (LiChrosolv), methanol (gradient grade), sodium chloride (NaCl) and tris(hydroxymethyl)-aminomethane (Tris) were obtained from Merck (Darmstadt, Germany). Ammonia solution (33%) was from Riedel-de Haën (Seelze, Germany). Ammonium hydrogen carbonate (ultrapure) and trifluoroacetic acid (TFA; for protein sequence analysis) were purchased from Sigma–Aldrich (Steinheim, Germany). Water for non-HPLC purposes was produced by a Milli-Q UF Plus ultrafiltration device from Millipore (Eschborn, Germany).

The enzyme inhibitor Pefabloc® SC (4-(2-aminoethyl-)benzene-sulfonyl fluoride hydrochloride) was purchased from Biomol (Hamburg, Germany). The inhibitors 4-amidinophenylmethanesulfonyl fluoride hydrochloride (APMSF), bis(*p*-nitrophenyl) phosphate sodium salt (BNPP), copper(II) sulfate (99%), ethylenediamine tetraacetic acid disodium salt dihydrate (EDTA; 99%), ethyleneglycol-bis(2-aminoethylether)-tetraacetic acid (EGTA; 98%), *N*-α-tosyl-L-lysine chloromethylketone hydrochloride (TLCK), sodium fluoride (NaF; 99%), tetrabutylammonium bromide (TBA; 99%) and zinc sulfate heptahydrate (99%) were from Sigma–Aldrich (Steinheim, Germany).

2.2. Rat serum

Male and female Wistar rats (*Rattus norvegicus*, Crl:WI (Han)), weighing between 155 and 265 g, were delivered by Charles River (Sulzfeld, Germany). They were housed and fed in accordance with institutional and regulatory guidelines. Blood was drawn from the rats by exsanguination *via* the caudal vena cava under anesthesia using isofluran (Forene®; Abbott, Wiesbaden, Germany). Serum was pooled from both males and females, equivalently in volume, and stored frozen for not longer than 14 days at approximately −20 °C.

Table 1
HPLC conditions for the inhibition studies and drug assays of native serum and the isolated fractions.

	Sagopilone		Drospirenone				MPA		
HPLC columns (all from VDS Optilab, Berlin, Germany)	Kromasil 100 C18, 5 μm, 250 × 4.6 mm		Hypersil ODS 120 Å, 3 μm, 125 × 4.6 mm				Spherisorb ODS-2, 3 μm, 125 × 4.6 mm		
HPLC eluents	• Eluent 1: acetic acid 0.1 % (v/v) • Eluent 2: ACN		• Eluent 3: acetic acid 0.1 % (v/v), pH 7.5, adjusted with ammonia solution • Eluent 4: methanol				• Eluent 5: ACN/water 30%/70% + 0.15% (v/v/v) acetic acid • Eluent 6: ACN/water 70%/30% + 0.15% (v/v/v) acetic acid		
	(min)	1	2	(min)	3	4	(min)	5	6
HPLC gradient of the eluents 1 and 2, 3 and 4, or 5 and 6 (%, v/v) over run time (min)	0	80	20	0	75	25	0	100	0
	8	40	60	35	45	55	20	0	100
	20	40	60	55	0	100	25	0	100
	23	20	80	58	0	100	35	100	0
	25	20	80	60	75	25			
	27	80	20						

2.3. Dog serum

Male and female Beagle dogs (*Canis familiaris*), weighing between 6.2 and 9.0 kg, were delivered by Marshall BioResources (North Rose, USA). They were housed and fed in accordance with institutional and regulatory guidelines. Blood was drawn from the jugular vein and processed to serum. Serum was pooled, equivalently in volume, from both males and females. It was used immediately or stored for not longer than one day at approximately 4 °C.

2.4. Inhibition studies of native serum

Initial incubation experiments in rat and dog serum have shown that drospirenone and MPA were both degraded in rat and dog serum whereas sagopilone was cleaved in rat serum only. To find efficient enzyme inhibitors, selected esterase and protease inhibitors (see Section 2.1) were tested for their potency to prevent degradation of sagopilone, drospirenone, and MPA in rat serum as well as of MPA and drospirenone in dog serum. The selection of enzyme inhibitors was mainly based on their reported solubility and stability in water (or buffered solutions), their inhibitory properties (e.g. irreversible inhibitors were preferred over reversible ones), their toxicity, and their inexpensive availability since these inhibitors would be used in serum (or even blood) samples during preclinical and clinical studies.

All the chosen inhibitors were dissolved in water, apart from EGTA which was prepared in a Tris–HCl-buffered solution (100 mM) containing 10 mM $CaCl_2$ (pH 7.4), due to the higher solubility of EGTA in this buffer. Ninety microlitre of native serum were pre-incubated with 10 μL of the inhibitor solution for 30 min in a water bath at 37 ± 0.5 °C. This mixture was then given to one of three drugs (whose solutions in ethanol had been dried before under a gentle stream of nitrogen) and incubated for 2 min, 6 h or 20 min (referring to sagopilone, drospirenone or MPA in rat serum, respectively) or for 6 h (referring to drospirenone or MPA in dog serum) at 37 ± 0.5 °C. The final concentrations (and specific radioactivity) of the drugs were as follows: 0.15 μM (580 GBq/mmol) for sagopilone, 10 μM (17 GBq/mmol) for drospirenone, and 0.2 μM (832 GBq/mmol) for MPA in rat serum, respectively, and 0.2 μM (832 GBq/mmol) or 1 μM (83 GBq/mmol) for MPA, and 10 μM (17 GBq/mmol) for drospirenone in dog serum, respectively. The final concentrations of the inhibitors are given in Tables 2 and 3. Corresponding positive controls were prepared using 10 μL of a Tris–HCl-buffered solution (100 mM), containing 10 mM $CaCl_2$ (pH 7.4), instead of the inhibitor solution. The reaction in each sample was stopped by adding 100 μL ACN and vortexing. After centrifugation, the supernatants were dried under a gentle stream of nitrogen. The residues were dissolved in 100 μL of eluent 1, 3 or 5 (referring to sagopilone, drospirenone or MPA, respectively; see Table 1) and stored at 10 °C for subsequent HPLC analysis. Twenty microlitre of each sample were injected onto the column. Table 1 summarizes the drug-specific chromatography conditions. The HPLC system consisted of a P680A HPG-2 pump (set at 1 mL/min), an ASI-100T autosampler (set at 10 °C), a TCC-100 MSV column oven (set at 22 °C), an UVD 170U ultraviolet–visible spectroscopy (UV–vis) detector (all from Dionex Softon, Germering, Germany), and a radiochemical detector (Radiomatic 525TR; Packard Instrument, Meriden, USA) using Ultima-Flo M (PerkinElmer, Shelton, USA) as liquid scintillation cocktail. The chromatograms were evaluated by use of Chromeleon 6.50 software (Dionex Softron, Germering, Germany). The peak identity of the drugs and their cleavage products was assessed in former internal studies by co-chromatography with the reference compounds. The quantitation of the drugs and their corresponding cleavage products was accomplished by determining the ratio of the peak area of the parent compound to the cleavage product(s) as measured by radiochemical detection. One sample per drug and inhibitor concentration was analyzed. The ratio of the relative peak area of the cleaved drug in the inhibited sample to that of the cleaved drug in the positive control sample was calculated. The difference between this ratio and 100% was defined as inhibition (in %).

2.5. Esterase activity assay using NPA

General esterase activity of the isolated fractions from the purification steps (see Section 2.8 and Fig. 2) was determined by a kinetic assay using the non-specific esterase substrate NPA which we have already used in our previous study [38]. Two hundred microlitres of each fraction were mixed with 10 μL of a NPA solution (50 mM; in methanol) in wells of a microplate (Nunc F96; Nunc, Wiesbaden, Germany). Then, the change of the absorbance was measured every 15 s over 3 min at a wavelength of 405 nm in a spectrophotometer (SpectraMax 190; Molecular Devices, Sunnyvale, USA) set at 30 °C. The measurement was evaluated by SoftMax Pro software (version 4.7; Molecular Devices) giving the maximum slope of a linear regression curve from the absorbance values between the fixed time points. Blank samples (using purification buffers only instead of fractions) were measured. Fractions with higher maximum slopes than those of the respective blank samples were further considered for PAGE analyses (see Section 2.10), protein determination (see Section 2.9), drug assays (see Section 2.6), and the next step of the purification sequence (see Section 2.8).

2.6. Esterase activity assay using sagopilone, drospirenone, or MPA

The fractions from the purification steps (see Section 2.8) were washed with a Tris–HCl-buffered solution (100 mM, pH 7.4) containing 10 mM $CaCl_2$. Then, the washed fractions were concentrated by using Amicon Ultra-15 ultrafiltration device (MW cut-off: 3 kDa; Millipore, Cork, Ireland). One hundred microlitre of the concentrated fractions, or native serum, were added to the given drug (whose solution in ethanol had been dried under a gentle stream of nitrogen) and incubated in a water bath at 37 ± 0.5 °C. The final concentrations (and specific radioactivity) of the drugs in the incubation mixtures were as follows: 3 μM (55 GBq/mmol) for sagopilone, 10 μM (17 GBq/mmol) for drospirenone, and 0.2 μM (832 GBq/mmol) for MPA in rat or dog serum, respectively. The incubation procedure and HPLC analyses were done as described for the inhibition studies (see Section 2.4). The time periods for incubation in native, or fractions from, rat serum were 0, 1, 5, 30, and 60 min for sagopilone (serum), 0, 5, and 60 min for sagopilone (fractions), 0, 10, 30, 60, and 120 min for drospirenone (serum), 0, 1, and 4 h for drospirenone (fractions), 0, 1, 5, 10, and 20 min for MPA (serum), and 0, 5, and 10 min for MPA (fractions). The time periods for incubation in native, or fractions from, dog serum were 0, 1, 2, 4, and 6 h for sagopilone (serum), 0, 1, 3, 6, and 12 h for drospirenone (serum), 0, 6, and 10 h for drospirenone (fractions), 0, 1, 4, 8, and 12 h for MPA (serum), and 0, 6, and 10 h for MPA (fractions). One sample per drug, incubation time point and pooled fraction, or serum type, was analyzed. Using the relative peak areas (which were calculated as described for the inhibition studies), the amount of the parent compound (in pmol) was calculated with the help of the known initial drug concentration in the incubation mixture. The amounts of the parent compounds were correlated with the incubation time. The activity (in μU) was defined as the greatest slope of the linear regression curves between the fixed time points in accordance with the data evaluation of the NPA assay (see Section 2.5). One unit (U) of activity represents the amount of enzyme hydrolyzing 1 μmol of drug in 1 min under assay conditions.

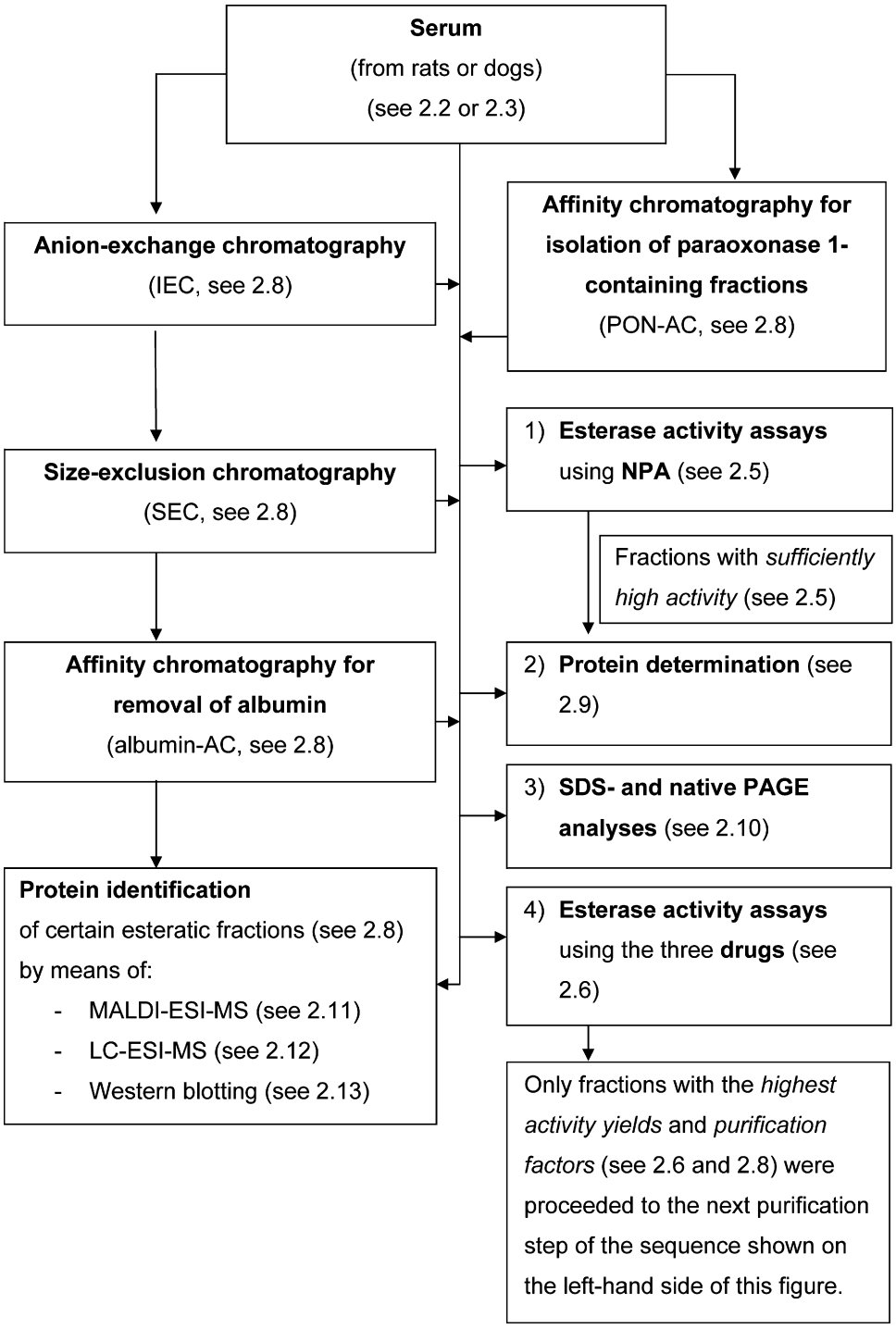

Fig. 2. Flow chart of the purification procedure of esteratic fractions from rat and dog serum with keys to applied esterase activity assays and other analytical techniques.

2.7. Buffers used for the purification steps

The buffers A, B, and C were used for the anion-exchange chromatography step (see Section 2.8). Buffer A consisted of a Tris–HCl-buffered solution (20 mM) containing 10 mM NaCl and 10% (v/v) glycerine (pH 7.6). Buffer B was a Tris–HCl-buffered solution (20 mM) containing 200 mM NaCl and 10% (v/v) glycerine (pH 7.6). Buffer C was a Tris–HCl-buffered solution (20 mM) containing 500 mM NaCl and 10% (v/v) glycerine (pH 7.6).

Buffer D, used for size-exclusion chromatography (see Section 2.8), consisted of a Tris–HCl-buffered solution (20 mM) containing 150 mM NaCl and 10% (v/v) glycerine (pH 7.6).

The buffers E and F were used for affinity chromatography removing albumin (see 2.8). Buffer E was a Tris–HCl-buffered solution (20 mM) containing 10% (v/v) glycerine (pH 7.0). Buffer F consisted of a Tris–HCl-buffered solution (20 mM) containing 1.5 M NaCl and 10% (v/v) glycerine (pH 7.0).

The buffers G, H, and I were prepared for the affinity chromatography isolating PON1-containing fractions (PON-AC, see Section 2.8). Buffer G was a Tris–HCl-buffered solution (20 mM) containing 2 M NaCl, 1 mM $CaCl_2$, 5 μM EDTA, and 10% (v/v) glycerine (pH 8.0). Buffer H consisted of a Tris–HCl-buffered solution (20 mM) containing 1 mM $CaCl_2$, 5 μM EDTA, and 10% (v/v) glycerine (pH 8.0). Buffer I was a Tris–HCl-buffered solution (20 mM) containing

0.1% (w/v) sodium deoxycholate, 1 mM $CaCl_2$, 5 μM EDTA, and 10% (v/v) glycerine (pH 8.0).

All the buffers were filtered and degassed prior to use.

2.8. Isolation and purification of esteratic fractions

The isolation and purification scheme comprised three sequential steps (methods I–III, see below and Fig. 2) which were mainly orientated on the method of Tsuji et al. [19] with some alterations [39]. In addition, PON1-containing fractions from dog serum were obtained by method IV (i.e. PON-AC; see below) essentially based on the procedure of Gan et al. [40]. All chromatographic steps were carried out on the protein purification system ÄKTAprime plus (GE Healthcare, Uppsala, Sweden) at approximately 4 °C, except from the methods III and IV which were accomplished at ambient temperature. The runs were monitored by an integrated UV detector measuring at 280 nm.

2.8.1. Anion-exchange chromatography (IEC)

Frozen, pooled rat serum was thawed at ambient temperature. An aliquot was assayed for total protein content (see Section 2.9) and esterase activities with regard to the three drugs (see Section 2.6). Six millilitre of serum were diluted with buffer A to 75 mL. An anion-exchange chromatography column (DEAE Sephacel matrix filled in an XK 26/20 column, GE Healthcare, Uppsala, Sweden) was loaded with the prepared serum. Throughout the run, a constant flow rate of 2 mL/min was used. After washing with buffer A, the proteins eluted from the column during a linear gradient from 10 to 200 mM NaCl (buffer A and B, respectively). Then, the column was washed by a one-step gradient from 200 to 500 mM NaCl (buffer B and C, respectively). Finally, the starting conditions were restored (buffer A). Fractions (10 mL each) were collected during the run. The fractions with sufficient esterase activity (as measured by the NPA assay; see Section 2.5) were further used for PAGE analyses and protein determination. Based on PAGE results, qualitatively similar fractions were pooled for the drug assays. Pooled fractions with the highest drug-specific activity yields and purification factors were prepared for the next purification step (see Fig. 2).

2.8.2. Size-exclusion chromatography (SEC)

The active fractions from the IEC were washed with buffer D and concentrated. An XK 26/70 column was packed with Sephacryl S-300 HR (GE Healthcare, Uppsala, Sweden). After sample application, elution was carried out at 0.5 mL/min with approximately 300 mL of buffer D, collecting fractions of 5 mL volume. The fractions were analyzed in the same way as after the IEC step. Those with the highest drug-specific activities were chosen to be prepared for the next purification step.

For determination of molecular mass, the following standard proteins were used to calibrate the column (Gel Filtration Standard; Bio-Rad Laboratories, Hercules, USA): bovine thyroglobulin (670 kDa), bovine γ-globulin (158 kDa), chicken ovalbumin (44 kDa), horse myoglobin (17 kDa), and vitamin B_{12} (1.35 kDa).

2.8.3. Affinity chromatography (AC) for removal of albumin (albumin-AC)

The pooled fractions from the SEC were washed with buffer E by ultrafiltration and concentrated. An affinity chromatography column filled with 5 mL of a Cibacron Blue F3G-A-coupled matrix (HiTrap Blue HP; GE Healthcare, Uppsala, Sweden) was employed for removal of albumin. Following sample application, the column was washed with the NaCl-free buffer E. Then, the bound proteins were eluted by a one-step gradient from 0 to 1.5 M NaCl (buffer E and F, respectively). Eventually, the starting conditions were restored (buffer E). Fractions (2 mL each) were collected and taken for analysis in the same way as after the IEC step. The fractions from rat or dog serum exhibiting the highest activity for sagopilone or MPA cleavage were subjected to protein identification (see Section 2.11 or Section 2.12), respectively.

Applying the purification sequence on dog serum esterases resulted in a total activity loss of the SEC fractions and albumin-AC fractions as determined by the NPA assay (see Section 2.5). This was in marked contrast to the IEC fractions from dog serum as well as to all of the fractions from rat serum. Therefore, all the SEC fractions as well as albumin-AC fractions from dog serum with UV absorbance values greater than that of buffer D as well as buffer E or F, respectively, were further used for PAGE analyses (see Section 2.10), protein determination (see Section 2.9) and drug assays (see Section 2.6).

2.8.4. Affinity chromatography (AC) for isolation of PON1-containing fractions (PON-AC) from dog serum

To isolate PON1-containing fractions from dog serum, a dye-ligand affinity chromatography was carried out using a 5-mL column filled with a Cibacron Blue F3G-A-coupled matrix (HiTrap Blue HP; GE Healthcare, Uppsala, Sweden). One millilitre of dog serum was diluted with buffer G to 2 mL and applied to the column. After washing, elution of PON1-containing fractions was carried out by a one-step gradient from 2 to 0 M NaCl (buffer G and H, respectively). The remaining PON1-containing fractions were eluted off the column by changing from buffer H to buffer I. Then, the initial conditions were restored with buffer G. Fractions (2 mL each) were collected and assayed for protein concentration (see Section 2.9). In addition, all the fractions which made part of a peak in the UV chromatogram were taken for sodium dodecyl sulfate-(SDS-)PAGE analysis with Western blot detection (see Section 2.13).

2.9. Protein determination

Protein concentration was determined with the bicinchoninic acid (BCA) assay [41]. Referring to the IEC fractions, 10 μL were mixed with 200 μL of the BCA working solution (BCA Protein Assay Kit; Thermo Scientific, Rockford, USA) and incubated for 30 min at 37 °C. As the SEC, albumin-AC, and PON-AC fractions usually contained less than 200 μg/mL protein, the micro-BCA assay was employed [42]. Therefore, 100 μL were mixed with 100 μL of the micro-BCA working solution (Micro BCA Protein Assay Kit; Thermo Scientific) and incubated for 2 h at 37 °C. In both procedures, the standard curve was prepared from serial dilutions of bovine serum albumin.

2.10. Sodium dodecyl sulfate-(SDS-)PAGE and native (non-denaturating) PAGE

Two different PAGE techniques, i.e. SDS-PAGE and native PAGE, were used to reveal the purity of the fractions from the purification steps. It is known that both techniques differ in their separation parameters, thereby providing an improved distinction of qualitatively unequal fractions [43].

For SDS-PAGE analysis, NuPAGE 4–12% Bis–Tris gels were chosen (Invitrogen, Carlsbad, USA). Prior to loading, the samples were incubated with a buffer containing 2% (w/v) lithium dodecyl sulfate and 10% (v/v) 2-mercaptoethanol for 10 min at 70 °C. The gels were run in a buffer containing 50 mM 3-(*N*-morpholino)propane sulfonic acid and 0.1% (m/v) SDS at a constant voltage of 200 V for approximately 45 min. After the run, the proteins were stained by use of Coomassie Blue following the manufacturer's instructions (Colloidal Blue Stain Kit; Invitrogen).

Regarding native PAGE analysis, Novex 8–16% Tris–Glycine gels were employed (Invitrogen). The samples were mixed with a buffer containing 50 mM Tris–HCl and loaded onto the gels. The electrophoresis was carried out in a buffer containing 2.5 mM Tris and

19.2 mM glycine at a constant voltage of 125 V for approximately 90 min. Detection was carried out as mentioned before.

2.11. Protein identification by matrix-assisted laser desorption ionization (MALDI)–time of flight (TOF)–mass spectrometry (MS)

For protein identification, the 70-kDa protein band of a Coomassie-stained SDS-PAGE gel (from a sagopilone-cleaving albumin-AC fraction purified from rat serum) was cut out, minced and treated with a mixture (50%/50%; v/v) of ACN and a solution of 50 mM ammonium hydrogen carbonate. After decolorization and bleaching, the material was incubated with a solution containing 50 mM ammonium hydrogen carbonate and 10 ng/μL trypsin (Sequencing Grade Modified Trypsin; Promega, Mannheim, Germany) overnight at 37 °C. The tryptic digestion was stopped by adding 2% (v/v) TFA. The gel pieces were extracted using a mixture of water/ACN/TFA (39.9%/60%/0.1%; v/v/v) The supernatants were dried under vacuum, the residues then re-dissolved in 0.1% (v/v) TFA and used for MALDI-TOF-MS analysis with a matrix containing α-cyano-4-hydroxycinnamic acid (α-CHCA Matrix Kit; LaserBio Labs, Sophia-Antipolis, France). The analysis was carried out on 4700 Proteomics Analyzer (Applied Biosystems, Foster City, USA) and evaluated by Mascot software (Matrix Science, London, UK). The peptides were identified by matching the resulting MS and MS/MS spectra to reported proteins in the NCBI database (National Center for Biotechnology Information, Bethesda, USA). To estimate the probability of a false-positive match, "protein scores" of greater than 67 were considered significant for an actual match ($p < 0.05$) [44].

2.12. Liquid chromatography (LC)–electrospray ionization (ESI)–mass spectrometry (MS)

For protein identification, the 140-kDa protein band of the SDS-PAGE gel (from an MPA-cleaving albumin-AC fraction purified from dog serum) was cut out and minced. The gel pieces were incubated with a mixture (50%/50%; v/v) of acetonitrile (ACN) and a solution of 50 mM ammonium hydrogen carbonate. After centrifugation, decolorization and bleaching, the material was incubated with a solution containing 50 mM ammonium hydrogen carbonate and 10 ng/μL trypsin (Sequencing Grade Modified Trypsin; Promega, Mannheim, Germany) at 37 °C. The tryptic digestion was stopped by adding 2% (v/v) trifluoroacetic acid (TFA). After extraction with a mixture of water/ACN/TFA (39.9%/60%/0.1%; v/v/v), the supernatants were united, dried, and re-dissolved in 0.1% (v/v) TFA. Ten microlitre were injected into the 4000 Q TRAP LC/MS/MS system (linear ion trap) (Applied Biosystems, Foster City, USA). The LC was carried out on the Eksigent NanoLC-1Dplus system (Eksigent Technologies, Dublin, USA) using an Acclaim PepMap column (stationary phase: C18; 15 cm, 75 μm i.d., particle size: 5 μm, pore size: 300 Å) (Dionex, Sunnyvale, USA) with a C18-Trap column (Agilent, Waldbronn, Germany). Two eluents were prepared: (A) 0.1% (v/v) formic acid and (B) ACN / water / formic acid (95%/4.9%/0.1%; v/v/v). A typical LC gradient was run as follows (in % [v/v]): 0–5 min: A/B 95/5; 5–30 min: A/B from 95/5 to 60/40; flushing the column with A/B >60/<40; restoration of the initial conditions (A/B 95/5). Data evaluation was carried out using Mascot software (Matrix Science, London, UK). Matching of the peptides and estimation of the probability of a false-positive match was done as described before (see Section 2.11).

2.13. Western blot

To directly detect PON1 in the PON-AC fractions from dog serum, SDS-PAGE analysis of these fractions as well as of dog serum was carried out as described in Section 2.10, except from protein staining. After electrophoresis, the gel was blotted onto a nitrocellulose membrane (iBlot Gel Transfer Stacks, Regular; Invitrogen, Carlsbad, USA) according to the manufacturer's instructions [45]. Then, the membrane was incubated with blocking buffer (i.e. a solution of 0.05 M Tris-buffered saline (TBS) containing 0.05% (w/v) Tween 20 and 5% (w/v) bovine serum albumin (pH 8.0) (all ingredients from Sigma–Aldrich, Steinheim, Germany). The primary antibody (anti-paraoxonase 1 antibody, produced in rabbit; #P0123, Sigma–Aldrich, Steinheim, Germany) was diluted 1:1000 (v/v) in blocking buffer and added to the membrane. After washing, the secondary antibody (anti-rabbit IgG [whole molecule], alkaline phosphatase conjugate, developed in goat; #A7778, Sigma–Aldrich, Steinheim, Germany) was diluted 1:3000 (v/v) in blocking buffer and incubated with the membrane. Following washing, a solution of nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Premixed BCIP/NBT Solution; Sigma–Aldrich, Steinheim, Germany) was added to visualize PON1-containing protein bands. Negative control samples using solutions of AChE (from Electrophorus electricus [electric eel]), BChE (from horse serum), porcine liver esterase (all from Sigma–Aldrich, Steinheim, Germany), and protein-free SDS-PAGE sample buffer were analyzed either.

Table 2
Inhibition of drug cleavage in native rat serum by different enzyme inhibitors. Inhibition (in %) was defined as the difference between 100% and the ratio of the relative peak area of the hydrolyzed drug in the inhibited sample to that of the hydrolyzed drug in the positive control (which contained buffer instead of inhibitor solution). For experimental conditions, see 2.4.

Inhibitor (concentration)	Inhibition (%)		
	Sagopilone	Drospirenone	MPA
BNPP (1 mM)	97.4	64.0	90.7
BNPP (5 mM)	95.4	55.0	94.8
EDTA (20 mM)	26.5	67.6	2.1
NaF (10 mM)	74.2	n.d.[a]	n.d.
NaF (100 mM)	92.7	50.6	56.5
Pefabloc® SC (1 mM)	92.1	61.8	92.3
Pefabloc® SC (10 mM)	89.8	66.1	93.2
TBA (10 mM)	87.1	n.d.	n.d.
TBA (100 mM)	95.8	99.4	22.2
TLCK (10 mM)	82.9	19.1	n.d.
Zinc sulfate (10 mM)	19.8	0.0	54.9

[a] n.d.: not determined.

2.14. Evaluation of kinetic data

All data from the inhibition studies (see Section 2.4) as well as from the drug assays (see Section 2.6) were computed and evaluated using SigmaPlot software (version 10.0) in connection with SigmaStat (version 3.5) and Enzyme Kinetics Modul (version 1.3) (all from Systat Software, Richmond, USA).

3. Results

3.1. Inhibition studies of native rat serum

The serine hydrolase inhibitors BNPP and Pefabloc® SC were highly effective inhibitors of sagopilone and MPA cleavage in rat serum (>90% inhibition; Table 2). Drospirenone degradation was less influenced by these inhibitors. In contrast, the metallohydrolase inhibitor EDTA had a greater effect on drospirenone than on sagopilone or MPA cleavage. NaF was a more efficient inhibitor of sagopilone cleavage compared to the cleavage of the other drugs whereas TBA equally inhibited sagopilone and drospirenone hydrolysis. TLCK sufficiently prevented sagopilone but not drospirenone cleavage. Zinc sulfate was a weak inhibitor of MPA hydrolysis, showing virtually no effect on the cleavage of the two other drugs.

Table 3
Inhibition of drug cleavage in native dog serum by different enzyme inhibitors. For definition of inhibition (in %) see Table 2; for experimental conditions, see Section 2.4.

Inhibitor (concentration)	Inhibition (%)	
	Drospirenone	MPA
APMSF (10 mM)	58.7	75.8
BNPP (5 mM)	31.2	70.2
EDTA (20 mM)	95.6	37.2
EGTA (10 mM)	95.4	30.4
Copper(II) sulfate (1 mM)	73.6	30.0
NaF (100 mM)	94.2	9.6
Pefabloc® SC (10 mM)	78.9	82.3
TBA (100 mM)	93.9	32.8
TLCK (10 mM)	75.0	88.1
Zinc sulfate (1 mM)	69.4	2.5

3.2. Inhibition studies of native dog serum

Referring to MPA cleavage in dog serum, the serine hydrolase inhibitors Pefabloc® SC, APMSF, BNPP, and TLCK were found to be highly efficient inhibitors (70–88% inhibition; Table 3) whereas all the other tested inhibitors exhibited no relevant inhibition. With regard to drospirenone stabilization, the metallohydrolase inhibitors EDTA and EGTA were as efficient as the enzyme inhibitors NaF and TBA (94–95% inhibition each; Table 3). Pefabloc® SC (exhibiting 79% inhibition) was more efficient than APMSF (59% inhibition) or BNPP (no marked inhibition) in preventing drospirenone cleavage. The PON inhibitors copper sulfate and zinc sulfate as well as TLCK revealed high inhibition (69–75%) concerning drospirenone hydrolysis.

3.3. Purification of esteratic fractions from rat serum and protein identification by MALDI-TOF-MS

The isolation and purification of esteratic fractions from rat serum by use of a sequential, three-step purification protocol was successful in enriching the drug-specific esterase activities. This is demonstrated by high purification factors ranging from 83 to approximately 10,000 (Table 4). Especially, the last purification step (i.e. an affinity chromatography for removal of albumin, referred to as albumin-AC) greatly increased the specific activities of the fractions. The first purification step (i.e. an anion-exchange chromatography, referred to as IEC), however, resulted in a great loss of esteratic activities giving low activity yields in the end of the purification process. Referring to sagopilone, the pooled fractions with the highest activity from the first purification step eluted on the descending part of a peak maximum (corresponding to approximately 70 kDa or less) in the size-exclusion chromatography (referred to as SEC; chromatograms not shown). The activity for sagopilone cleavage was clearly separated from the proteins which were bound to the Cibacron Blue F3G-A-coupled matrix (chromatogram not shown). Consequently, the esterases cleaving sagopilone did not bind to this dye-ligand affinity column. The bound proteins mainly consisted of serum albumin as revealed by SDS-PAGE (results not shown). With respect to drospirenone and MPA, the chromatograms and their activity distributions were comparable to those of sagopilone (data not shown). Thus, the major esteratic activities concerning the three drugs were not associated with serum albumin.

The SDS-PAGE of the albumin-AC fractions cleaving the three drugs unveiled several protein bands (Fig. 3), indicating that the fractions were only partially purified. The Coomassie-stained protein bands of the SDS-PAGE gel of the active fractions hydrolyzing sagopilone were investigated by protein identification using tryptic digestion and MALDI-TOF-MS. At approximately 70 kDa (Fig. 3A), we were able to match the individual MS/MS spectra of two peptides (GNWAHLDQLAALR and LGIWGLFSTGDEHSR) of this protein band to the rat CE isoenzyme ES-1 (NCBI no. gi|2506388). The protein score of 144 for this match was significant ($p < 0.05$). We found, however, that rat serum albumin (NCBI no. gi|12402861) was also present in this protein band. Although not investigated, the finding that a protein band at approximately 68 or 70 kDa occurred in the SDS-PAGE gels of the drospirenone- or MPA-cleaving fractions, respectively, suggested the existence of ES-1 and albumin in these fractions either (Fig. 3B and C).

3.4. Purification of esteratic fractions from dog serum and protein identification by LC-ESI-MS

Sagopilone was not degraded in dog serum within the incubation time (results not shown). We therefore only investigated the esterases cleaving MPA and drospirenone by protein isolation and identification techniques.

The three-step purification protocol used for isolation of the esterases cleaving MPA resulted in an activity yield of approximately 12% with 645-fold purification (Table 5). The pooled fractions with the highest activity from the first purification step (IEC) eluted at the first, small peak maximum of the SEC corresponding to molecular masses of above 484 kDa (chromatograms not shown). The esterases were firmly bound to the Cibacron Blue affinity column (used for albumin-AC) and only recovered using a high-salt buffer (chromatogram not shown). With the help of the albumin-AC, the specific activity of the MPA-cleaving fractions was strongly enhanced as compared to both preceding purification steps (Table 5).

This purification scheme was not successful referring to drospirenone-cleaving esterases since the found esteratic activity of the active IEC fractions was completely lost after the SEC step (results not shown). PON1 was thus assumed to be involved in drospirenone cleavage. The PON-AC yielded three groups of PON1-containing fractions which sequentially eluted from the column by washing with a NaCl-free buffer, elution with sodium deoxycholate, and, finally, restoration of the starting conditions with a high-salt buffer (chromatogram not shown). The main PON1 pro-

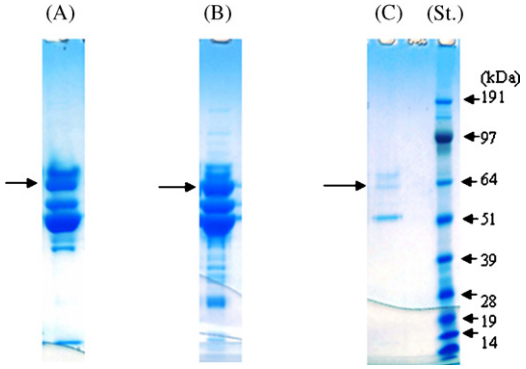

Fig. 3. SDS-PAGE gels of the albumin-AC fractions from rat serum cleaving sagopilone (A), drospirenone (B), and MPA (C). Lane A, B, and C contained approximately 4.9, 8.1, and 0.2 μg protein, respectively. Detection was accomplished by Coomassie Blue staining (see Section 2.10). The lane designated by "(St.)" shows the bands of the following molecular-mass standard proteins (See Blue Plus2 Pre-Stained Standard; Invitrogen, Carlsbad, USA): myosin (191 kDa), phosphorylase (97 kDa), bovine serum albumin (64 kDa), glutamic dehydrogenase (51 kDa), alcohol dehydrogenase (39 kDa), carbonic anhydrase (28 kDa), myoglobin red (19 kDa), and lysozyme (14 kDa). Two other standard proteins (aprotinin and insulin, B chain) exhibited bands below 14 kDa but were only poorly resolved. The arrows in gel A, B, and C indicate protein bands at 70, 68, and 70 kDa, respectively. The 70-kDa band in gel A was excised and subjected to tryptic digestion and, subsequently, MALDI-TOF-MS (see Section 2.11). Thereby, the rat carboxylesterase isoenzyme ES-1 (contaminated with rat serum albumin) was found.

Table 4
Summary of purification factors and activity yields of sagopilone-, drospirenone-, and methylprednisolone aceponate- (MPA-)cleaving fractions from rat serum. One unit (U) of activity is defined as the amount of enzyme cleaving 1 μmol of drug in 1 min under assay conditions (see Section 2.6). Specific activity was calculated by dividing the total activity by the total protein content of the fractions from the distinct purification steps. Purification factors were obtained by the ratio of the specific activity of the fractions to the specific activity of rat serum. Activity yields give the ratio of the total activity of the fractions to that of serum.

	Purification step	Fraction volume (mL)	Total activity (μU)	Total protein (mg)	Specific activity (μU/mg)	Purification factor (fold)	Activity yield (%)
Sagopilone	Rat serum	6	255	510	0.50	1.0	100.0
	IEC	30	30.5	44.6	0.69	1.4	12.0
	SEC	15	43.1	18.5	2.33	4.7	16.9
	Albumin-AC	4	11.4	0.05	236	472	4.5
Drospirenone	Rat serum	6	236	510	0.52	1.0	100.0
	IEC	30	12.5	42.3	0.30	0.6	5.2
	SEC	25	12.8	15.9	0.80	1.8	5.4
	Albumin-AC	4	3.4	0.08	42.9	82.5	1.4
MPA	Rat serum	6	49.6	501	0.10	1.0	100.0
	IEC	20	14.7	6.74	2.18	21.8	29.6
	SEC	15	5.0	0.56	9.00	90.0	10.1
	Albumin-AC	4	1.2	1.24 μg	996	9956	2.4

Table 5
Summary of purification factors and activity yields of drospirenone- and methylprednisolone aceponate- (MPA-)cleaving fractions from dog serum. For definition of one unit (U) of activity, specific activity, purification factors, and activity yields, see Table 4.

	Purification step	Fraction volume (mL)	Total activity (μU)	Total protein (mg)	Specific activity (μU/μg)	Purification factor (fold)	Activity yield (%)
MPA	Dog serum	6	0.87	331	2.63	1.0	100.0
	IEC	50	0.22	19.6	11.4	4.3	25.6
	SEC	35	0.13	0.68	192	73.1	14.9
	Albumin-AC	22	0.10	0.06	1697	645	11.8
MPA	Dog serum	1	0.15	59.6	2.44	1.0	100.0
	PON-AC	114	0.03	1.91	16.7	6.9	22.0
Drospirenone	Dog serum	1	6.30	59.6	105	1.0	100.0
	PON-AC	114	0.14	1.79	80.9	0.7	2.3

tein band was located at approximately 41 kDa referring to the three fraction pools (Fig. 4, gel 1, lanes B to D) as well as to native dog serum (Fig. 4, gel 1, lane A) as detected by Western blot. Some minor bands were found below the 41-kDa band (Fig. 4, gel 1, lane B and C). All the other PON-AC fractions and the negative control samples did not reveal any protein bands by Western blot (results not shown). The three groups of PON-AC fractions which were demonstrated to contain PON1 were pooled to determine their esteratic activity towards drospirenone and MPA (see 2.6). The activity yield concerning drospirenone cleavage was relatively low (2%) and the specific esterases were not enriched by PON-AC (Table 5). In contrast, the isolation and purification of the MPA-cleaving esterases by PON-AC were more successful as found by a relatively high activity yield (22%) and a purification factor of 7 (Table 5).

The SDS-PAGE gel of the MPA-cleaving albumin-AC fractions revealed some protein bands, indicating that the fractions were only partly purified (Fig. 4, gel 2, lane E). The Coomassie-stained protein bands of this gel were further investigated by protein identification using tryptic digestion and LC-ESI-MS technique. We were able to match the peptide mass fingerprint (obtained by MS) of the digested peptides of the 140-kDa band to canine α_2-

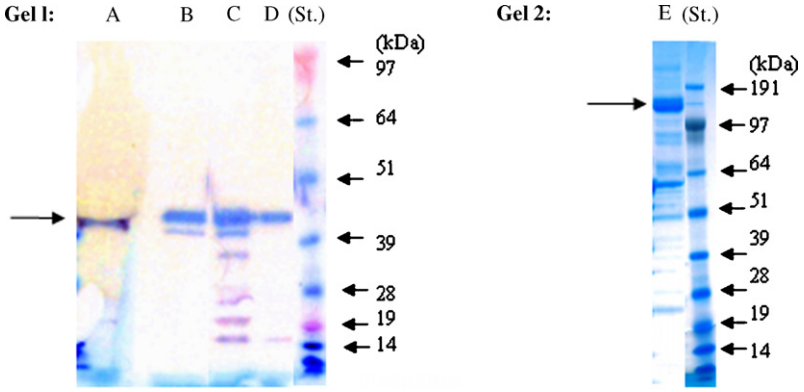

Fig. 4. Gel 1: SDS-PAGE gel of native dog serum (lane A) and the three fraction pools (lanes B–D) from the PON-AC of dog serum. Lane A, B, C, and D contained 224, 51, 69, and 40 μg protein, respectively. At approximately 41 kDa (see arrow), PON1 was detected in the lanes A to D by Western blot using an anti-PON1 antibody (see Section 2.13). Gel 2: SDS-PAGE gel of one active albumin-AC fraction from dog serum cleaving MPA (lane E). Lane E contained 1.1 μg protein. The protein band at approximately 140 kDa (see arrow) was identified as α_2-macroglobulin by tryptic digestion and subsequent LC-ESI-MS (see Section 2.12). The lane designated by "(St.)" shows the bands of the molecular-mass standard proteins as given in Fig. 3. Among them, myosin (191 kDa) was lost on gel handling concerning gel 1. In gel 1, the pre-stained standard proteins were visually detected. In gel 2, detection was accomplished by Coomassie Blue staining (see Section 2.10).

macroglobulin (α_2M; NCBI no. gi|73997689). The protein score of 1941 for this match was significant ($p < 0.05$). The sequence coverage (i.e. the percentage of the theoretical amino acid sequence of the peptide found in the detected peptide sequence) amounted to approximately 20% while 38 peptides were matched.

4. Discussion

The aim of the present study was to study the stability of three structurally different and clinically relevant drug ester compounds in rat and dog serum and to find, where necessary, effective protection against the esteratic cleavage with the help of selected enzyme inhibitors. The investigated compounds were sagopilone (a macrocyclic lactone), drospirenone (a steroidal γ-butyrolactone), and MPA (a steroid with open-chain ester groups). To better understand the different efficiency of the tested inhibitors towards the drug-cleaving enzymes, we were interested in the identity of these enzymes. Therefore, we partly purified the esteratic fractions from serum of each species. As a first result, the isolation and purification of esteratic fractions from rat serum enabled us to identify ES-1 in the sagopilone-cleaving fractions. The purification of dog serum esterases led, furthermore, to the identification of α_2M in the fractions cleaving MPA. Likewise, PON1 was found in the drospirenone-cleaving fractions from dog serum. The found effective inhibitors can now be used for stabilization of these drugs in serum *ex vivo*.

4.1. Inhibition studies of native rat serum

Referring to the inhibition studies in native rat serum (Table 2), BNPP and Pefabloc® SC exhibited high efficacy for inhibition of sagopilone and MPA cleavage whereas drospirenone hydrolysis was less influenced. BNPP and Pefabloc® SC act as phosphorylating and sulfonylating agents, respectively, known to primarily inhibit serine hydrolases [18,46,47]. Therefore, we assume that serine hydrolases were mainly involved in the cleavage of sagopilone and MPA. Due to the lower, but still distinct capability of BNPP and Pefabloc® SC concerning drospirenone stabilization, this drug was probably *not only* cleaved by serine hydrolases but also by hydrolases without an active serine residue in their catalytic sides, such as PON. PON is a calcium-dependent hydrolase cleaving organophosphates (without being inhibited thereby) [48]. Moreover, sulfonyl fluorides are known to faintly inhibit PON [49]. The metal-ion chelating agent EDTA has been reported to strongly inhibit PON by complexation of calcium ions which are absolutely required for the activity and stability of PON [13,50]. In context with the relatively high efficiency of EDTA to stabilize drospirenone (as compared to the other drugs), we assume that PON was mainly responsible for the cleavage of drospirenone.

Although sagopilone- and MPA-cleaving enzymes were all equally sensitive towards serine hydrolase inhibitors, EDTA was able to discriminate between the drug-specific esterases. The lower efficiency of EDTA for sagopilone stabilization (as compared to MPA) let us assume that sagopilone was cleaved by CE whereas MPA was subjected to BChE activity. This was deduced from other studies reporting that EDTA was a more efficient inhibitor of BChE than of CE [51,52].

Zinc sulfate was partly efficient for the stabilization of MPA. The inhibition of BChE and some serine proteases by zinc ions has been reported [53–57]. Therefore, we assume that these hydrolases were mainly involved in MPA cleavage. However, it is also known that BChE inhibited by zinc ions can be partly reactivated in the presence of calcium ions (which were included in our pre-incubation buffer) [55,58]. Having this in mind, it could not be excluded that BChE was also responsible for sagopilone and drospirenone cleavage even though the stabilizing effect of zinc ions on these drugs was minor in our study.

TLCK is a histidine-modifying agent which generally forms covalent bonds with serine residues in serine hydrolases leading to irreversible inhibition [46]. In contrast to CE, it has been reported that BChE was only reversibly inhibited by TLCK [19,51,59,60]. As TLCK had virtually no effect on drospirenone or MPA cleavage, we assume that BChE was involved in these degradations. Sagopilone hydrolysis was markedly inhibited by TLCK so that BChE was probably not responsable for this cleavage.

Summarizing the results from the inhibition studies, we assume that sagopilone was hydrolyzed by CE whereas BChE was responsible for drospirenone and MPA cleavage. Moreover, drospirenone was cleaved by PON while serine proteases were involved in MPA cleavage.

4.2. Inhibition studies of native dog serum

We found that sagopilone was not degraded in dog serum within the applied incubation time (see Section 2.6). This is in contrast to the results from rat serum where sagopilone was rapidly cleaved. We thus conclude that dog serum does not contain the CE that we assumed to be responsible for sagopilone cleavage in rat serum. This result is in accordance with others who reported a much greater CE activity as well as a high CE concentration in rat serum compared to dog serum as revealed by incubation and gel electrophoresis studies [15,16,27,30,61].

To effectively prevent MPA and drospirenone from enzymatic cleavage, selected enzyme inhibitors were studied in native dog serum. The serine hydrolase inhibitors Pefabloc® SC, APMSF, and BNPP exhibited marked inhibition of MPA degradation in dog serum (Table 3), thus pointing to the involvement of serine esterases in MPA cleavage.

Concerning drospirenone, there are good reasons to conclude that PON1 was involved in drospirenone hydrolysis as shown by the inhibition studies (Table 3). EDTA and EGTA are both known metal-ion chelating agents capable of mainly inhibiting metal-ion dependent enzymes (metallohydrolases) such as PON1.

The serine hydrolase inhibitors APMSF, BNPP, and Pefabloc® SC were less efficient than EDTA or EGTA, precluding any relevant involvement of serine hydrolases in drospirenone cleavage [46].

The high potency of NaF may be explained by precipitation of calcium fluoride due to the presence of calcium ions (in the buffer solution we used), leading to a markedly reduced calcium concentration and, subsequently, PON1 inhibition.

TBA was highly efficient in stabilizing drospirenone. As a possible reason, we assume that TBA (as a surface-active agent [62]) affected PON1 in such a way that this interaction resulted in conformational changes and inhibition of the enzyme as described for other detergents [63–65].

The great inhibitory effect of zinc and copper(II) ions on drospirenone cleavage also points to a major role of PON1 in this context [50,66]. This is supported by the finding of our study in rat serum where drospirenone cleavage was partly ascribed to rat serum PON1 (see Section 4.1). Furthermore, the structurally related spironolactone was also reported to be cleaved by PON isoenzymes [67,68].

4.3. Purification of esteratic fractions from rat serum and protein identification

Aiming to confirm our first suggestions on the identity of the drug-specific esterases and to understand the different inhibitor sensitivity, we partly purified the active fractions from rat serum to identify the responsible enzymes.

The applied three-step purification protocol to isolate and purify esterases from rat serum proved successful as shown by the high final purification factors (Table 4). Using an IEC as the first purification step was mainly based on the method of Tsuji et al. [19]. The direct application of serum to an anion-exchange column was additionally described by others [69,70]. Some reports on the isolation of esterases from rat serum or plasma employed fractionated precipitation with ammonium sulfate, followed by IEC [71–73]. We excluded the precipitation as this working step would mean a further physical burden on the proteins due to ultrafiltration or dialysis prior to IEC. The subsequent gel filtration (SEC) enabled us to estimate the molecular size of the drug-cleaving esterases to approximately 70 kDa or less (chromatogram not shown). By means of a dye-ligand affinity chromatography for removal of albumin (albumin-AC) using a Cibacron Blue F3G-A-coupled matrix, we obtained fractions greatly enriched in esteratic activities concerning the cleavage of the three drugs. The main esteratic activities were recovered in the flow-through fractions from this affinity chromatography (chromatogram not shown). This was in complete accordance with other reports on esterase isolation showing that BChE and CE did not bind to Cibacron Blue [30,74]. Due to the overall success of the purification sequence and the found activity distributions in the albumin-AC, we hypothesize that esterases, presumably BChE and/or CE, were involved in the cleavage of the drugs. Moreover, even serine proteases could be present in the active fractions owing to their ability to form tight complexes with BChE, being stable throughout isolation [75,76]. The low activity yields but high purification factors that we found were also experienced by others using an IEC as one of the first purification steps applied to esterases [19,30,71]. SDS-PAGE analyses of the active albumin-AC fractions revealed several protein bands (Fig. 3). The purification procedure could probably be improved to achieve fractions with higher purity than we did. For instance, affinity chromatography using specific antibodies or inhibitor ligands as well as immunoprecipitation may be useful as shown in other purification schemes [69,72,74,77,78].

As a first approach to protein identification, we identified the proteins of the SDS-PAGE gel of the albumin-AC fractions cleaving sagopilone. By tryptic digestion and MALDI-TOF-MS of the protein bands, we found the rat CE isoenzyme ES-1 contaminated with serum albumin in the band at approximately 70 kDa (Fig. 3 A). Both proteins were probably also present in the corresponding 68-kDa or 70-kDa protein band in the SDS-PAGE gels of the albumin-AC fractions cleaving drospirenone or MPA, respectively (Fig. 3 B and C). ES-1 is a member of the CES1C isoenzyme family [79]. CES1 isoenzymes are the main CE species in rat serum [72,79]. However, an isoenzyme belonging to the CES2 family has been detected in rat plasma, but only in a minor concentration [80]. Serum albumin that we identified either is known for its esterase-like activity [7,9,14]. However, it was not involved in the cleavage of the three drugs studied here since the albumin-containing fractions from albumin-AC eluted by the high-salt buffer did not exhibit relevant drug-cleaving activities (data not shown). From these results, we postulate that rat ES-1 could principally be involved in the cleavage of sagopilone (and probably both other drugs). Consistent with other reports on rat serum CE [30,72,74,77], we found a molecular mass of rat ES-1 of approximately 70 kDa by SEC (chromatogram not shown) as well as SDS-PAGE (Fig. 3A). It should be noted that we have yet only identified the *main* proteins in the bands of the SDS-PAGE gels. Therefore, our findings did not exclude that other esterases, especially BChE and serine proteases, were also present in the active fractions.

In all, our first hypotheses from the inhibition studies of native serum were supported by the results from protein isolation and identification. Summarizing the results from the protein purification, we conclude that the presence of serine hydrolases (i.e. CE, BChE, serine proteases) in the active fractions was likely. In fact, ES-1 was subsequently identified in the esteratic fractions cleaving sagopilone. Although the identification of drospirenone- and MPA-cleaving esterases and further studies on the identity of sagopilone-cleaving esterases remain to be carried out, we assume that sagopilone was cleaved by CE, drospirenone by BChE and PON while MPA was degraded by BChE and serine proteases. These assumptions would also explain the inhibitor sensitivity since, for instance, the assumed enzyme for sagopilone cleavage (i.e. ES-1) would be greatly inhibited by serine hydrolase inhibitors (BNPP, Pefabloc® SC) as we found here indeed.

Regarding drospirenone, it has been reported that the structurally related drug spironolactone was cleaved by human PON1 and PON3, thus supporting our suggestion of the involvement of PON in drospirenone cleavage [67,68]. As PON3 has solely been found in rat hepatic microsomes yet, we assume that rat PON1 was responsible for this hydrolysis in serum [68,81]. With regard to structural MPA analogues, the charged steroid-21-monoester methylprednisolone hemisuccinate was mainly cleaved by a rat CES2 isoenzyme [80], thus assuming different substrate specificities of rat BChE and CE isoenzymes. Indeed, the uncharged 21-monoester methylprednisolone acetate, but not the hemisuccinate ester, was described to be hydrolyzed by human BChE [82] which supports our assumption of the involvement of BChE in MPA cleavage. To our best knowledge, studies of enzymes cleaving sagopilone or structurally related compounds have not yet been published.

4.4. Purification of esteratic fractions from dog serum and protein identification

For isolation and identification of the MPA-specific esterases from dog serum, we applied a three-step purification scheme as outlined before (see Section 4.3). The purification cascade was successful concerning MPA-cleaving esterases as shown by the high final purification factor (Table 5). We found α_2M at 140 kDa (Fig. 4, gel 2, lane E) by protein identification using LC-ESI-MS of the SDS-PAGE gel of one active albumin-AC fraction. The endogenous protease inhibitor α_2M has no reported *intrinsic* esterase activity. We therefore assume that α_2M formed a complex with a serine protease or serine esterase, but retaining the capability of the bound protease or esterase to cleave MPA [83,84]. The protease inhibitor α_2M is a homotetramer with a molecular mass of 180 kDa for each monomer which can be fragmentated into smaller species upon sample preparation for SDS-PAGE [84,85]. We thereby explain the found molecular mass of 140 kDa in the present study. This complex seemed to be stable throughout the purification steps since the main MPA-cleaving activity eluted in the SEC in the range of high molecular mass proteins (>484 kDa). Moreover, it is known that α_2M strongly binds to Cibacron Blue-coupled matrices, thereby explaining the activity distribution of the albumin-AC fractions [86,87].

With respect to the identification of MPA-specific serine esterase, we hypothesize that BChE was involved in this cleavage. This is mainly based on our results from rat serum (see Section 4.3) as well as on the finding that the structural analogue methylprednisolone-21-acetate was reported to be cleaved by this enzyme [82]. In addition, our hypothesis is in full accordance with the high efficiency of the serine hydrolase inhibitors Pefabloc® SC, APMSF, and BNPP. It was, moreover, reported that BChE can complex with α_2M while retaining its esterase activity even on native PAGE gels [7]. BChE can, however, not alone be responsible for MPA cleavage because, in this case, one would have seen much greater inhibition by NaF and lower inhibition by TLCK in the presence of specific substrates than we actually did [59,88]. It has been described that BChE possesses two functionally different enzymatic sites; one is responsible for the BChE esterase activity while

the other is for the aryl acylamidase activity [75,89,90]. Both sites are overlapping but not identical as found by studies of the influence of certain enzyme inhibitors and activators on these functions [69,90,91]. The two enzymatic activities are, however, inhibited by serine hydrolase inhibitors [30,69]. Although the effects of NaF and TLCK on the amidase function remain to be investigated, we assume that the amidatic site of BChE was responsible for MPA cleavage. This hypothesis is based on the fact that the found influence of both inhibitors was in contrast to what would have been expected for their influence on the esterase function of BChE. MPA is, of course, not an amide but both enzymatic sites of BChE are in close contact and possess a catalytically active serine residue, thus probably enabling the amidatic site to readily cleave esters such as MPA [90,92].

Further evidence for the involvement of PON1 in drospirenone cleavage in dog serum came from the purification methods we applied, thus supporting the results from the inhibition studies. Being successful for isolation of MPA-cleaving enzymes, the three-step purification procedure, however, resulted in a complete activity loss after the SEC step concerning the enzymes hydrolyzing drospirenone. Due to our hypothesis of the involvement of PON1, we used a relatively specific method for isolation of PON1 using Cibacron Blue affinity chromatography (PON-AC) which was basically described by Gan et al. [40]. We obtained several fractions being part of peaks in the UV chromatogram (not shown). These fractions were investigated by SDS-PAGE and, subsequently, Western blotting using an anti-PON1 antibody. In contrast to Gan et al. [40], we found by Western blot that PON1 already eluted from the affinity column due to washing with a NaCl-free buffer. The remaining esterase was then desorbed from the column by elution with sodium deoxycholate and restoration of the initial conditions. The early desorption of PON1 from the column was probably due to the lower NaCl concentration that we used as compared to that of Gan et al. (2 M *vs.* 3 M NaCl) [40]. Therefore, PON1 might not have been firmly adsorbed to the matrix and, as a consequence, it was more readily desorbed. We had to substitute the buffer containing 3 M NaCl (used by Gan et al. [40]) by a buffer containing 2 M NaCl since our purification system did not tolerate the high viscosity of the former buffer. We detected PON1 in the SDS-PAGE gel of native dog serum as well as of the mentioned PON-AC fractions at approximately 41 kDa (Fig. 4, gel 1, lanes A–D) which fully corresponds to other reported molecular masses of serum PON (laying in the range of 37–48 kDa). [93–95]. The presence of some minor PON1 bands below the main 41-kDa band in lane B and C (Fig. 4) could be explained by the fact that PON1 may exist in different glycosylated isoforms [93,94]. To our best knowledge, we here report for the first time the detection of PON1 in dog serum although its presence has long been suggested owing to incubation and gel electrophoresis studies with PON substrates [27,29,96,97]. The amino acid sequence of dog PON1 has been deduced from the corresponding complementary deoxyribonucleic acid (cDNA) sequence [98]. The primary antibody that we used for Western blot (see Section 2.13) was raised against a synthetic peptide of 12 amino acids representing a part of the sequence of human PON1. It is known that anti-PON antibodies exhibit cross-reactivity towards other PON isoenzymes within a given species and even between different species due to the high intra- and interspecific sequence homology of PON isoenzymes (reaching 60–86%) [13,98–100], clearly explaining the positive (cross-)reaction towards dog serum PON1 in our study.

We used the three mentioned groups of PON1-containing fractions from the PON-AC (see Section 3.4) as one pool to determine their esteratic activity towards drospirenone or MPA. However, the activity yield and purification factor of these fractions concerning drospirenone cleavage were low as compared to MPA (Table 5). One reason for this finding may be the fact that the cleaved lactone ring of drospirenone may tend to re-cyclize in aqueous solutions as described for other γ-butyrolactones [101,102]. PON1 generally exhibits lower specific activities towards lactones compared with aromatic non-cyclic esters [67,103]. In addition, dog serum was reported to have lower catalytic efficiencies towards aromatic esters in comparison to rat serum, for instance [29,104]. All these factors might have resulted in a true and/or artificial low activity of the PON1-containing fractions in terms of drospirenone cleavage.

The PON1-containing fractions from PON-AC gave a high activity yield and considerable enrichment of esterases cleaving MPA as compared to drospirenone (Table 5). Although we have demonstrated that PON1 was present in these fractions (see above), PON1 was not assumed to be responsible for MPA cleavage. This hypothesis is mainly based on the finding that PON1 was able to attack estradiol-3,17β-diesters cleaving the ester group at position 3

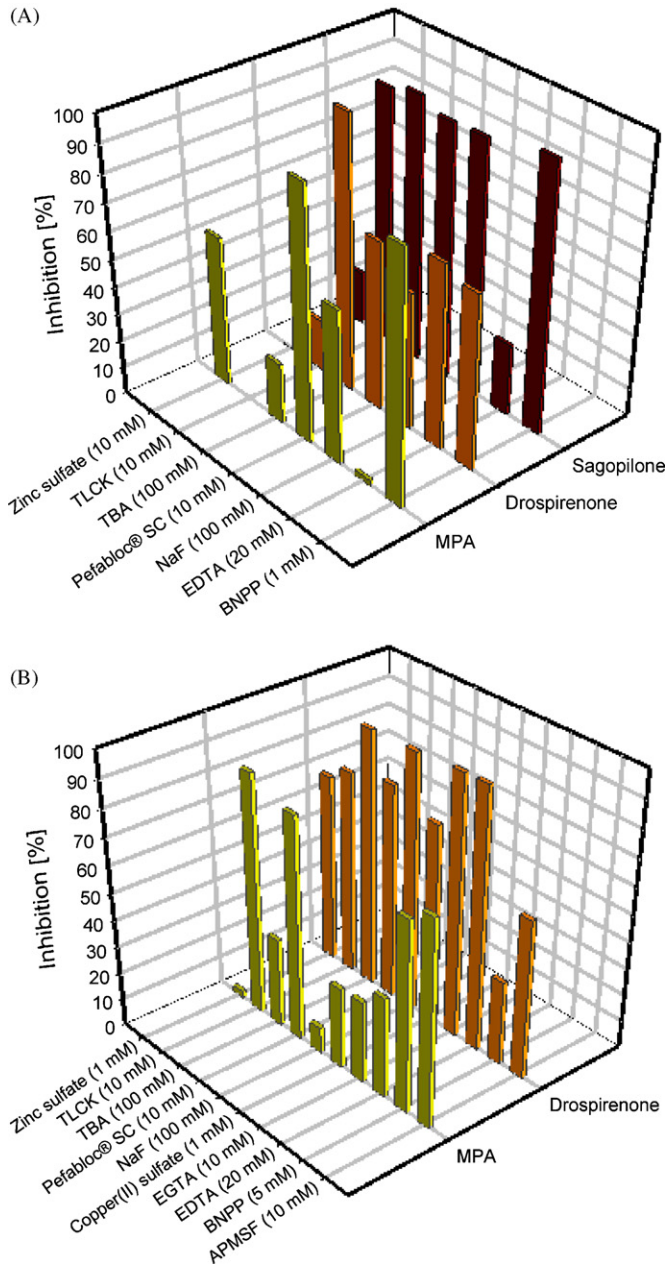

Fig. 5. Efficiency of selected enzyme inhibitors to prevent sagopilone, drospirenone, and MPA from esteratic degradation in native rat (A) or dog serum (B). Data were selected from Tables 2 and 3.

but not that one at position 17β (unless there was an adjacent enolization in ring D of the steroid structure) [105]. Moreover, methylprednisolone-21-acetate, a structural analogue to MPA, was found not to be cleaved by human PON1 [82]. Further reasons to conclude that PON1 did not cleave MPA can be brought about by our results from the inhibition studies (Table 3): The metallohydrolase inhibitors EDTA and EGTA had no relevant inhibitory effect on MPA cleavage. The high specific activity of the PON1-containing fractions towards MPA can probably be explained by the fact that other esterases may be isolated along with PON1 using Cibacron Blue affinity chromatography as reported, for instance, for platelet-activating factor acetylhydrolase (PAF-AH) [106,107].

In all, we assume that drospirenone was cleaved by dog serum PON1 whereas MPA was degraded by BChE.

5. Conclusion

To summarize, we successfully found efficient enzyme inhibitors for the stabilization of sagopilone, drospirenone, and MPA in rat serum as well as of drospirenone and MPA in dog serum. Moreover, the inhibitor sensitivity of the drug-specific esterases in native serum, in conjunction with the (first) results from protein purification and identification, shed some light on the identity of the cleaving esterases, thereby explaining the different efficiency of the tested inhibitors. Further studies are clearly needed to have sound evidence on the actual involvement of the assumed enzymes in drug cleavage.

Our results from the inhibition experiments (Tables 2 and 3, Fig. 5) can be used to stabilize these and structurally related compounds in rat and canine serum, and probably blood, samples *ex vivo* during preclinical studies. As a straightforward approach, one should extend the use of the found inhibitors to improve the stability of chemically unrelated drug esters in blood of other test species as well as in human blood for exploring the efficiency of the inhibitors in preclinical and clinical studies with newly developed drug ester compounds.

Conflict of interest

Matthias Koitka was a former research fellow at Bayer Schering Pharma AG, Berlin, Germany. Joachim Höchel and Hille Gieschen are full-time employees at Bayer Schering Pharma AG, Berlin, Germany. Hans-Hubert Borchert has no conflicts of interest to disclose.

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