Involvement of carboxylesterase 1 and 2 in the hydrolysis of mycophenolate mofetil

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Non-standard abbreviation

CES; carboxylesterase

MMF; mycophenolate mofetil

MPA; mycophenolic acid

HLC; pooled human liver cytosols

HLM; pooled human liver microsomes

HJC; pooled human jejunal cytosols

HIC; pooled human ileal cytosols

HIM; pooled human intestinal microsomes
Abstract

Mycophenolate mofetil (MMF) is the ester prodrug of the immunosuppressant agent mycophenolic acid (MPA), and is rapidly activated by esterases following oral administration. However, the role of isoenzymes in MMF hydrolysis remains unclear. While human plasma, erythrocytes, and whole blood contain MMF hydrolytic activities, the mean half-lives of MMF in vitro were 15.1, 1.58 and 3.20 hours, respectively. Thus, blood esterases appeared to contribute little to the rapid MMF disappearance in vivo. In vitro analyses showed that human intestinal microsomes exposed to 5 and 10 µM MMF exhibited hydrolytic activities of 2.38 and 4.62 nmol/min/mg protein, respectively. Human liver microsomes exhibited hydrolytic activities of 14.0 and 26.1 nmol/min/mg protein, respectively, approximately 6-fold higher than observed for intestinal microsomes. MMF hydrolytic activities in human liver cytosols were 1.40 and 3.04 nmol/min/mg protein, respectively. As hepatic cytosols generally contain 5-fold more protein than microsomes, MMF hydrolysis in human liver cytosols corresponded to approximately 50% of that observed in microsomes. Fractions obtained by 9,000 g centrifugation of supernatants from COS-1 cells expressing human carboxylesterase (CES) 1 or 2 exhibited MMF hydrolytic activity, with CES1-containing fractions
showing higher catalytic efficiency than CES2-containing fractions. The CES inhibitor bis-\(p\)-nitrophenylphosphate inhibited MMF hydrolysis in human liver microsomes and cytosols with IC\(_{50}\) of 0.51 and 0.36 \(\mu\)M, respectively. In conclusion, both intestinal and hepatic CESs may be involved in MMF hydrolysis, especially CES1, and play important roles in MMF bioactivation. Hepatic CES1 activity levels may help explain the between-subject variability observed for MMF utilization.
Introduction

Mycophenolic acid (MPA), the active metabolite of mycophenolate mofetil (MMF), is a non-competitive and reversible inhibitor of inosine monophosphate dehydrogenase, and therefore inhibits the de novo pathway of guanine nucleotide synthesis. Because T- and B-lymphocytes are dependent on de novo purine synthesis for proliferation, MPA has a cytostatic effect on lymphocytes [Allison and Eugui, 1993].

MMF was designed to improve MPA bioavailability as an ester-type oral prodrug. Oral bioavailability of MPA following MMF administration is reported to be 80.7% to 94% [Pescovitz et al. 2000; Bullingham et al. 1998; Staatz and Tett 2007], such that MMF is essentially completely hydrolysed to MPA by esterases [Bullingham et al. 1998]. Because MMF was almost completely absent in plasma from renal transplant patients [Bullingham et al. 1996; Armstrong et al. 2005], the conversion from MMF to active form MPA is considered to occur rapidly in the blood, intestine and liver, following gastrointestinal absorption [Lee et al. 1990].

Esterases in the blood, intestine and liver play important roles in drug metabolism and detoxication [Satoh et al., 2002]. Butylcholinesterase in plasma, arylesterase and carboxylesterase (CES)1 in the erythrocyte cytosol, and acetylcholinesterase in the
erythrocyte plasma membrane have been described [La et al., 1971; Ott et al., 1975; Oertel et al., 1983]. Human intestine and liver tissues express mainly CES2 and CES1/CES2, respectively, and CES expression in both the intestine and liver is extremely high compared with other organs [Satoh et al., 2002]. CESs play central roles in the hydrolysis and bioactivation of various drugs, including cocaine, heroin and CPT-11 [Pindel et al, 1997; Slatter et al, 1997]. In addition, CES1 and CES2 expressed in the liver cytosol are reported to be required for the bioactivation of CPT-11 and capecitabine [Xu et al., 2002; Tabata et al., 2004a, Tabata et al., 2004b].

Glucuronidation is an important phase II metabolic pathway for MPA, and the molecular mechanisms underlying this process have been demonstrated by various researchers [Bernard et al., 2004; Picard et al., 2005]. However, although we recently reported that MMF underwent hydrolysis as a phase I pathway via CES located the hepatic microsome fraction in humans [Fujiyama et al., 2009], the systemic mechanisms responsible for MPA formation have not yet been fully elucidated. Also, it is possible that inter-individual variations in MPA oral bioavailability might be due to phase I metabolism by CESs. Therefore, to clarify the mechanisms of systemic MMF hydrolysis, we characterized the MMF hydrolytic pathway using human whole blood, erythrocytes, plasma, tissue microsomes and cytosolic fractions. In addition, we
investigated the involvement of CES-family enzymes in MMF hydrolysis using tissue fractions from CES1- and CES2-expressing COS-1 cells.
Materials and methods

Reagents and Chemicals.

MMF and MPA were supplied by Roche Pharmaceuticals (Palo Alto, CA). 2-(6-methoxy-2-naphthyl) propenoic acid (naproxen), diisopropyl fluorophosphates (DFP), bis-p-nitrophenylphosphate (BNPP), and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma (St. Louis, MO, USA). Dulbecco’s modified Eagle’s medium, fetal calf serum, and Lipofectamine plus reagent were obtained from Life Technologies (Tokyo, Japan). All other chemicals and solvents were of the highest grade commercially available.

Enzyme sources.

Blood samples were collected into tubes containing EDTA from Japanese healthy volunteers (2 males and 3 females aged 23-59 years) (samples HB-1 to -5). Informed consent was obtained from all subjects. After taking an aliquot of whole blood, erythrocytes and plasma were separated by centrifugation at 3,000 rpm for 5 min. Erythrocytes were washed using an equal volume of saline. All samples were assayed on the same day as collection, and no hemolysis was observed.
Pooled human liver cytosols (HLC), pooled human intestinal microsomes (HIM) and pooled human liver microsomes (HLM) were purchased from GENTEST Corporation (Woburn, MA, USA). Pooled human jejunal cytosols (HJC) and human ileal cytosols (HIC) were purchased from Becton, Dickinson and Company (BD; Franklin Lakes, NJ, USA).

Expression of each human carboxylesterase isoform in COS-1 cells was carried out as previously described [Yamaori S et al, 1997]. Briefly, COS-1 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-incubated fetal calf serum. cDNA fragments encoding human CES1 or 2 were ligated into linearized mammalian expression vectors and transfected into COS-1 cells using Lipofectamine plus reagent according to the manufacturer’s instructions. After 48 h, the transfected cells were collected and sonicated to prepare cell lysates. The lysates were centrifuged at 9,000 g for 20 min and the resultant supernatants (S9 fractions) homogenized and kept at -80 ºC until assay. An S9 fraction from COS-1 cells transfected with a plasmid carrying CES2 cDNA ligated in the reverse orientation was used as a negative control. Protein concentrations were estimated using a BCA™ Protein Assay Kit (Takarabio, Shiga, Japan) with bovine serum albumin used as a standard.
Characterization of MMF hydrolysis in vitro.

All MMF hydrolysis assays were performed as previously described, with some modifications [Yamaori et al. 2006, Fujiyama et al. 2009].

Briefly, 450 µl aliquots of each human blood fraction (whole blood, erythrocytes or plasma) were preincubated at 37 ºC for 5 min. Reactions were initiated by the addition of 50 µl MMF solution (final concentration 100 µM). Incubations were carried out at 37 ºC for 0, 10, 45, 90, and 135 min, and terminated by the addition of 500 µl acetonitrile. To determine the kinetic parameters of MMF hydrolysis, MMF (5-1,600 µM) was incubated with or without each human tissue fraction (25 µg HIM protein, 20 µg HIC protein, 20 µg protein, 25 µg HLM protein, 50 µg HLC protein, or 25 or 50 µg S9 fraction protein obtained from COS-1 cells expressing human CES1 and CES2, respectively) in 100 mM sodium phosphate buffer (pH 7.4) to give a final volume of 0.5 ml in 5 ml test tubes. Mixtures were incubated using a shaking water bath at 37 ºC for 30 min for intestinal fractions (HIM HIC and HJC), 5 min for HLM, 15 min for HLC, or 10 and 45 min for S9 fractions from COS-1 cells expressing human CES1 and CES2, respectively. Reactions were terminated by immersion in an ice bath and the addition of 0.5 ml acetonitrile. Before extraction, naproxen (1.0 µg) in dimethylsulfoxide (10 µl) was added to the reaction mixtures as an internal standard, the solutions vortexed for
30 sec, and then centrifuged for 10 min at 3,000 g. Aliquots (10 μl) of the clear supernatants were filtered through Millipore filters (0.45 μm, Millex-LH®, Japan). The final concentration of organic solvent in all reaction mixtures was <1.0%.

Samples were then directly injected into a high-performance liquid chromatography apparatus (AS-2057 plus intelligent sampler, PU-2080 plus chromatography pump, UV-2075 plus ultraviolet detector, 807-IT integrator, JASCO, Tokyo, Japan) equipped with a CAPCELL PAK C18 MG II (250 mm × 4.6 mm, 5 μm, Shiseido, Tokyo, Japan). The mobile phase was 0.5% KH₂PO₄ (pH 3.5):acetonitrile (1:1). Elution was performed at a flow rate of 0.5 ml/min. MPA was monitored at a wavelength of 254 nm, and the lower limit of MPA detection was 25 ng/ml.

In preliminary experiments, reaction conditions were confirmed to ensure initial rates of MPA formation. MPA enzymatic production rates were deducted from non-enzymatic production rates obtained after incubation without added enzyme sources.

Inhibition studies.

Inhibitory effects on the hydrolysis of MMF to MPA were evaluated using the inhibitors DFP, BNPP and PMSF. Inhibitors were preincubated with human tissue fractions at
37 °C for 1 min to give final inhibitor concentrations of 0.001-1 µM (DFP), 0.01-100 µM (BNPP) and 0.1-1000 µM (PMSF). Reactions were started by the addition of MMF solution, and the MMF levels quantitated in reactions containing 12.5 µM liver fractions, 25 µM intestine fractions or S9 fractions from COS-1 cells expressing human CES1 or 2.

**Data analysis.**

MMF concentration–time data obtained from MPA production reactions were fitted to the equation \[ C(t) = A \cdot e^{-at} \] using SigmaPlot 9.0 software (HULINKS Inc., Tokyo, Japan). Velocity vs. MMF concentration plots were fitted to the Michaelis-Menten equation (one- or two-site saturation models) by nonlinear least-squares regression analysis using SigmaPlot 9.0 software (HULINKS Inc., Tokyo, Japan). IC\(_{50}\) values were calculated by nonlinear least-squares regression analysis using SigmaPlot 9.0 software.

**Statistical analysis.**

Statistical analyses were performed using an unpaired \( t \)-test or one-way analysis of variance followed by the Dunnet’s test using InStat 3 software (Graphpad Software, Inc.,
San Diego, CA, U.S.A.). A *P*-value of less than 0.05 was considered statistically significant.
Results

**MMF hydrolysis to MPA in human blood fractions.**

MMF hydrolysis in human blood samples was evaluated *in vitro*. MPA formation from non-enzymatic MMF hydrolysis after 10, 45, 90, and 135 min incubation were 0.35%, 1.11%, 2.02% and 3.15%, respectively, using 100 µM MMF in 100 mM phosphate buffer without the addition of enzyme sources. As shown in Fig. 2, whole blood, erythrocytes and plasma formed MPA from MMF time-dependently. The eliminative rate constants ($k_{el}$) of MMF in whole blood, erythrocytes and plasma were $0.24 \pm 0.11$, $0.46 \pm 0.11$ and $0.051 \pm 0.015$ h$^{-1}$, respectively, giving corresponding MMF half-lives ($t_{1/2}$) of $3.2 \pm 0.94$, $1.6 \pm 0.43$ and $15 \pm 6.1$ hr, respectively (Table 1). The half-life of MMF in whole blood was about 2-fold longer than that in erythrocytes, while the plasma MMF half-life was approximately 10-fold longer than that in erythrocytes.

**MMF hydrolysis to MPA in human tissue fractions.**

To calculate the kinetic parameters of MPA formation from MMF, MMF was incubated with HIM, HJC, HIC, HLM and HLC (Fig. 3). In preliminary experiments, reaction
conditions were confirmed to ensure that the initial rates of MPA formation were optimal for each protein concentration and each reaction time, as described in Materials and Methods. Velocity vs. MMF concentration plots for HIM, HJC and HIC are shown in Fig. 3A-C, with the three data plots best fitted to a two-site saturation model. Typical Eadie-Hofstee plots of MPA formed from MMF in the presence of HIM, HJC and HIC were clearly biphasic as shown in Fig. 3 inset. The kinetic parameters of MPA formation from MMF are shown in Table 2. For HIM, the estimated apparent $K_m$, $V_{max}$, and intrinsic clearance ($Cl_{int}$; $V_{max}/K_m$) values were 27.3 ± 4.7 µM, 15.6 ± 1.5 nmol/min/mg protein and 571 µl/min/mg protein, respectively, for the high affinity enzyme reaction and 782 ± 256 µM, 19.7 ± 1.2 nmol/min/mg protein and 252 µl/min/mg protein, respectively, for the low affinity enzyme reaction. For HJC, the estimated apparent $K_m$, $V_{max}$ and $Cl_{int}$ values for MPA formation were 14.9 ± 5.4 µM, 4.58 ± 0.72 nmol/min/mg protein and 307 µl/min/mg protein, respectively, for the high affinity enzyme reaction and 1091 ± 432 µM, 14.2 ± 1.76 nmol/min/mg protein and 13 µl/min/mg protein, respectively, for the low affinity enzyme reaction. For HIC, the estimated apparent $K_m$, $V_{max}$ and $Cl_{int}$ values for MPA formation were 17.5 ± 6.0 µM, 5.94 ± 0.84 nmol/min/mg protein and 339 µl/min/mg protein, respectively, for the high affinity enzyme reaction and 1656 ± 1355 µM, 12.0 ± 4.2 nmol/min/mg protein and 7 µl/min/mg protein, respectively, for the low affinity enzyme reaction.
mg protein, respectively, for the low affinity enzyme reaction.

Velocity vs. MMF concentration plots obtained using HLM and HLC are shown in Fig. 3D and E, respectively, and the data best fitted to a one-site saturation model. The estimated apparent $K_m$ and $V_{max}$ values for MPA formation using HLM were $992 \pm 69 \mu M$ and $3072 \pm 112 \text{ nmol/min/mg protein}$, respectively (Table 2). The $Cl_{int}$ of MMF hydrolysis for HLM ($3098 \text{ nl/min/mg protein}$) was higher than that reported in our previous study ($1308 \text{ nl/min/mg protein}$) [Fujiyama et al., 2009]. The estimated $K_m$, $V_{max}$ and $Cl_{int}$ values obtained for HLC were $329 \pm 20 \mu M$, $75.4 \pm 2.0 \text{ nmol/min/mg protein}$ and $230 \text{ nl/min/mg protein}$, respectively.

MMF hydrolytic activities for all tissue fractions are shown in Fig. 3F. MPA formation from 5 and 10 $\mu M$ MMF in the presence of HLM was $14.0$ and $26.1 \text{ nmol/min/mg protein}$, respectively, compared to $1.40$ and $3.04 \text{ nmol/min/mg protein}$, respectively, in the presence of HLC, $2.38$ and $4.62 \text{ nmol/min/mg protein}$, respectively, for HIM, $1.11$ and $1.92 \text{ nmol/min/mg protein}$, respectively, for HJC, and $1.29$ and $2.33 \text{ nmol/min/mg protein}$, respectively, for HIC. HLC corresponded to $10.0$ and $11.6\%$ of MMF hydrolytic activities of HLM with 5 and 10 $\mu M$ MMF, respectively.

**MMF hydrolysis by recombinant human CES1 and 2**
Levels of MPA formation in S9 fractions from COS-1 cells expressing human CES1 or CES2 following MMF incubation are shown in Fig. 4A. Hydrolytic activities on 100 µM MMF in response to the negative control, hCES1 and hCES2 were 0.51 ± 0.16, 83.6 ± 6.2 and 7.98 ± 0.43 nmol/min/mg protein, respectively. COS-1 CES1 and CES2 S9 fractions had significantly higher MMF hydrolytic activities compared with the negative control. To characterize MMF hydrolysis by CES1 and CES2, kinetic parameters were calculated from velocity vs. MMF concentration plots, as shown in Fig. 4B and C. In both cases, data best fitted to a one-site saturation model. The estimated $K_{\text{m}}$, $V_{\text{max}}$ and $Cl_{\text{int}}$ values for MPA formation were 225 ± 12 µM, 313 ± 5 nmol/min/mg protein and 1391 µl/min/ mg protein, respectively, for hCES1 and 22.3 ± 2.1 µM, 12.3 ± 0.2 nmol/min/mg protein and 552 µl/min/ mg protein, respectively, for hCES2 (Table 3).

Effects of CES inhibitors on MMF hydrolysis to MPA.

Inhibitory effects of DFP, BNPP and PMSF on MMF hydrolysis in the presence of HIM, HJC, HIC, HLM, HLC, hCES1 and hCES2 are shown in Fig. 5A to G. MPA formation from all enzymatic sources was inhibited by DFP, BNPP and PMSF in concentration-dependent manners, except for DFP and BNPP inhibition of MPA formation by HIM. Residual MMF hydrolytic activities in HIM in the presence of 10
µM DFP and 100 µM BNPP were 14.2% and 12.8% of control values, respectively (Fig. 4A). The estimated IC₅₀ values for inhibitory effects as determined from these residual activities are shown in Table 4. Inhibitory effects on MMF hydrolysis by DFP, BNPP and PMSF were stronger in HLC than in HLM, and the effects of DFP and BNPP were stronger on hCES2 than on hCES1.
Discussion

Several reports have found that the pharmacokinetics of MPA in humans is characterized by large inter- and intra-individual variability and that glucuronide activity from MPA may be involved in the between-individual variability [Kuypers et al. 2005; Baldelli et al. 2007]. In the present study, we focused on the importance of the MMF bioactivation pathway to MPA as a factor other than the MPA glucuronidation pathway previously researched.

At 10 minutes after peripheral intravenous infusion with MMF, plasma MMF concentrations fall below detection levels in human test subjects, which led to the conclusion that the half-life of MMF in human plasma \textit{in vivo} was approximately 2 minutes [Bullingham et al. 1996]. However, in the present \textit{in vitro} study, the estimated mean half-life of MMF in plasma was 15.1 hours, and 1.58 hours in erythrocytes. As the measured mean hematocrit value was 0.468 ± 0.053, esterases located in erythrocytes seemed to be involved in the MMF hydrolysis. However, the half-life of MMF in human blood \textit{in vitro} was much longer than that reported in a previous \textit{in vivo} study [Bullingham et al. 1996]. This suggests that blood esterases contribute little to the rapid disappearance of MMF in the human body, and that
gastrointestinal organs and the liver may be central to MMF hydrolysis.

Our study found that the order of MMF to MPA hydrolysis rate was liver > intestine, with HLM showing the highest MMF hydrolytic activity ($Cl_{int}$ for MPA formation in the presence of HLM being 3,098 nL/min/mg protein). However, our previous study gave an intrinsic clearance value for HLM of 1,308 nL/min/mg protein [Fujiyama et al., 2009]. The HLM used in the present and previous studies were supplied by BD and consisted of microsomes pooled from 33 and 27 donors, respectively. As microsomal carboxylesterase activity can exhibit large inter-individual variation, the difference in HLM activities between our studies could be due to chance differences in assay validation. The $Cl_{int}$ for MMF hydrolysis in response to enzymes sourced from intestinal tissues was 5-fold lower than that from liver cellular fractions. It is known that the intestine predominantly expresses the CES2 isoform rather than CES1 [Satoh et al., 2002; Taketani et al., 2007]. Therefore, it is possible that the MMF hydrolytic activity in hepatic fractions was mainly due to CES1, as the liver highly expresses both CES1 and CES2. To clarify the role of CES isoforms in MMF hydrolysis, we examined recombinant human cells expressing CES1 or CES2. Hydrolytic activity on 100 µM MMF by hCES1 was approximately 10-fold higher than that by hCES2, such that the $Cl_{int}$ for MPA formation by hCES1 was higher
than that by hCES2 (1391 vs. 552 nl/min/mg protein). Thus, it appears that CES1 may be important for MPA formation. However, as orally-administrated prodrugs encounter intestinal esterases before liver esterases, it is likely that some hydrolysis would still occur in the intestine.

Because CESs were reported to be expressed in the inner-lumen of the endoplasmic reticulum [Robbi and Beaufay, 1987; Medda and Proia, 1992], it was thought that only compounds located in the lumen underwent hydrolysis and glucuronidation. However, Tabata et al. reported that CES1 is present in both microsomes and the cytosols, and that cytosolic CES plays an important role in the bioactivation of the prodrug, capecitabine [Tabata et al. 2004a]. Because there is approximately 5-fold more total cytosolic protein than microsomal protein [Tabata et al. 2004a], when we extrapolated this to the results obtained in the present study, we estimated that cytosolic MMF hydrolysis was equivalent to about 37 or 88% of the microsomal MMF hydrolysis. This is consistent with our finding that HLC showed 46-50% lower MMF hydrolytic activity than HLM over the concentration range of 5-10 µM MMF. Based on these results, our study indicated that the order of MMF to MPA hydrolysis rates were HLM > HLC > HIM > HJC = HIC. Moreover, although MMF has high membrane permeability, MMF hydrolysis in the cytosol may be decreased by formation of MPA glucuronide...
metabolites by uridine diphosphate glucuronosyltransferases (UGTs) located in the inner-lumen of the endoplasmic reticulum, due to the low membrane permeability of MPA. Therefore, cytosolic CESs may be an important determinant of MMF pharmacokinetics in vivo.

We further investigated the esterases involved in MMF hydrolysis in human samples using a series of inhibitors. In erythrocytes, the CES inhibitor BNPP (10 µM) inhibited MMF hydrolysis by only 5.6% relative to control values, while the arylestease inhibitor 5, 5'-ditiobis(2-nitrobenzoic acid) showed no inhibition at all [Heymann and Krisch, 1967; Costello et al., 1983] (Supplement data). Therefore, an erythrocyte esterase other than CES and arylesterase, namely acetylcholinesterase, appeared to be responsible for MMF hydrolysis in whole blood. In intestinal fractions, addition of DFP and BNPP to HIM inhibited MMF hydrolysis by 87% relative to control values. Theoretically, 92% of the hydrolysis of 25 µM MMF by HIM was due to the high affinity enzyme present in HIM. The kinetic parameters of MPA formation by the high affinity enzyme in HIM were similar to the kinetics displayed by S9 fractions from COS-1 cells expressing CES2. Therefore, our results indicated that the HIM esterase involved in MMF hydrolysis is CES2. Although intestinal cytosolic fractions HJC and HIC also exhibited the biphasic curves, MMF hydrolysis in HJC and HIC were
completely inhibited by BNPP. This suggested that multiple CESs might be involved in this pathway. Moreover, to our knowledge, the existence of CESs in the intestinal cytosol has not been previously reported. As both microsomal and cytosolic CESs appear to play key roles in first-pass effects on the esterified prodrug, further study on intestinal cytosolic CESs is required.

In conclusion, MMF was hydrolyzed by human intestinal and hepatic fractions rather than by human blood fractions. Although orally-dosed MMF rapidly disappears from the blood, our results suggested that the disappearance of MMF was due mainly to hepatic CESs, especially CES1.
References


Footnotes

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Figure Legends

Fig. 1. Chemical structures of MMF and MPA

Fig. 2. Plots of MMF concentration as a function of incubation time with human blood fractions. Residual amounts of MMF were calculated by the formation of MPA in human blood fractions (whole blood, erythrocyte and plasma) at the indicated times. All points represent the mean ± SD of five subjects (HB-1 to 5).

Fig. 3. Velocity vs. concentration plots of MPA formation from MMF by gastrointestinal tissue fractions. MMF (5-1,600 µM) was incubated with HIM (A), HJC (B), HIC (C), HLM (D) or HLC (E) as described in Materials and Methods. Inset figures show Eadie-Hofstee plots. Vertical maximum scales in each graph are adjusted to enhance readability. (F) MMF hydrolytic activities at 5 µM (open columns) and 10 µM (closed column) for each enzyme source. Columns represent the mean of three determinations.

V: velocity, S: MMF concentration
Fig. 4. MMF hydrolysis catalyzed by S9 fractions from COS-1 cells expressing human CES1 and 2.

A; Hydrolysis of 100 µM MMF in S9 fractions from COS-1 cells expressing human CES1 and 2 were compared with a negative control. Columns represent the mean ± SD of three determinations. B and C; Velocity vs. MMF concentration plots of MPA formation from MMF by gastrointestinal tissue fractions. MMF (5-1,600 µM) was incubated with S9 fractions from COS-1 cells expressing human CES1 and 2 as described in Materials and Methods. Inset figures show Eadie-Hofstee plots. Vertical maximum scales in each graph are adjusted to enhance readability.

* Significantly different from the negative control, \( p<0.001 \).

Fig. 5. Effects of CES inhibitors on MPA formation by human gastrointestinal organ fractions and S9 fractions from COS-1 cells expressing CES1 and 2.

Inhibitory effects on MMF hydrolysis for each enzyme source were tested using DFP (●, 0.001 to 10 µM), BNPP (□, 0.01 to 100 µM) and PMSF (▲, 0.1 to 1000 µM). MMF concentration in each reaction mixture was 25 µM (intestinal fraction), 12.5 µM (liver fraction) and 25 µM (S9 fraction from COS-1 cells). Control activities were 7.42
(HIM), 2.66 (HJC), 3.02 (HIC), 23.3 (HLM), 4.36 (HLC), 29.3 (hCES1), and 5.50 (hCES2) nmol/min/mg protein. All points represent mean of two experiments.

**Fig. 6.** Schematic illustration of MMF bioactivation
Table 1. Kinetic parameters of MMF hydrolysis *in vitro* in human blood fraction.

<table>
<thead>
<tr>
<th>Sex</th>
<th>Plasma</th>
<th>Erythrocyte</th>
<th>Whole blood</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>kel</td>
<td>t&lt;sub&gt;1/2&lt;/sub&gt;</td>
<td>kel</td>
</tr>
<tr>
<td>HB1</td>
<td>M</td>
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<tr>
<td>HB2</td>
<td>F</td>
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<tr>
<td>HB3</td>
<td>M</td>
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<tr>
<td>HB4</td>
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<td>0.058</td>
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<tr>
<td>HB5</td>
<td>F</td>
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<td>11.3</td>
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<tr>
<td></td>
<td>Average</td>
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<tr>
<td></td>
<td>S.D.</td>
<td>0.015</td>
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kel: h<sup>-1</sup>, t<sub>1/2</sub>: h
### Table 2. Kinetic parameters MMF hydrolysis in human tissue fractions.

<table>
<thead>
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<th>Affinity</th>
<th>$V_{max}$</th>
<th>$K_m$</th>
<th>$Cl_{int}$</th>
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<tbody>
<tr>
<td>HIM</td>
<td>high</td>
<td>15.6  (3.0)</td>
<td>27 (9)</td>
</tr>
<tr>
<td></td>
<td>low</td>
<td>19.7  (2.4)</td>
<td>782 (512)</td>
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<tr>
<td>HJC</td>
<td>high</td>
<td>4.6    (1.4)</td>
<td>15 (10)</td>
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<tr>
<td></td>
<td>low</td>
<td>14.2   (3.6)</td>
<td>1091 (432)</td>
</tr>
<tr>
<td>HIC</td>
<td>high</td>
<td>5.9    (1.7)</td>
<td>18 (12)</td>
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<tr>
<td></td>
<td>low</td>
<td>12.0   (8.2)</td>
<td>1656 (2710)</td>
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<td></td>
<td>3073   (224)</td>
<td>992 (138)</td>
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<tr>
<td>HLM *</td>
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<td>1358   (58)</td>
<td>1038 (86)</td>
</tr>
<tr>
<td>HLC</td>
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<td>75     (4)</td>
<td>329 (40)</td>
</tr>
</tbody>
</table>

$V_{max}$: nmol/min/mg protein.

$K_m$: µM.

$Cl_{int}$ ($V_{max} / K_m$): nl/min/mg protein.

Values are represented as mean (SD).

*, kinetic parameters obtained from our previous report (Fujiyama et al, 2009).
Table 3. Kinetic parameters of MMF hydrolysis in S9 from COS-1 cells expressing CES1 and 2.

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<thead>
<tr>
<th></th>
<th>$V_{max}$</th>
<th>$K_m$</th>
<th>$Cl_{int}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>hCES1</td>
<td>313 (10)</td>
<td>225 (24)</td>
<td>1391</td>
</tr>
<tr>
<td>hCES2</td>
<td>12.3 (0.4)</td>
<td>22.3 (4.2)</td>
<td>552</td>
</tr>
</tbody>
</table>

$V_{max}$: nmol/min/mg protein  
$K_m$: µM  
$Cl_{int}$ ($V_{max}$ / $K_m$): nl/min/mg protein  
Values are represented as mean (SD).
Table 4. IC$_{50}$ of DFP, BNPP and PMSF for MMF hydrolysis.

<table>
<thead>
<tr>
<th></th>
<th>DFP (nM)</th>
<th>BNPP (µM)</th>
<th>PMSF (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIM</td>
<td>5.70$^a$</td>
<td>0.282$^a$</td>
<td>3.05</td>
</tr>
<tr>
<td>HJC</td>
<td>10.4</td>
<td>0.460</td>
<td>16.0</td>
</tr>
<tr>
<td>HIC</td>
<td>8.63</td>
<td>0.395</td>
<td>10.7</td>
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<tr>
<td>HLM</td>
<td>31.0</td>
<td>0.580</td>
<td>7.12</td>
</tr>
<tr>
<td>HLC</td>
<td>5.80</td>
<td>0.348</td>
<td>3.77</td>
</tr>
<tr>
<td>hCES1</td>
<td>10.4</td>
<td>0.330</td>
<td>2.10</td>
</tr>
<tr>
<td>hCES2</td>
<td>8.61</td>
<td>0.137</td>
<td>5.46</td>
</tr>
</tbody>
</table>

$^a$ IC$_{50}$ of DFP and BNPP were estimated as inhibition to the mean residual activity (1.00 nmol/min/mg protein) with 10 µM of DFP and 100 µM of BNPP.
Fig. 1
Fig. 2.

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A. HIM

Fig. 3A
B. HJC

Fig. 3B
C. HIC

Fig. 3C
Fig. 3D
E. HLC

Fig. 3E
Fig. 3F
Fig. 4A
B. hCES1

Fig. 4B
C. hCES2

Fig. 4C
A. HIM

Residual activity (% of control) vs. Inhibitor concentration (µM)

Fig. 5A
B. HJC

![Graph showing inhibition of activity with varying concentrations of inhibitor.]

Residual activity (% of control) vs. Inhibitor concentration (µM)

Fig. 5B
Fig. 5C

Inhibitor concentration (µM)

Residual activity (% of control)

C. HIC
Fig. 5D

Residual activity (% of control) vs. Inhibitor concentration (µM)

D. HLM
Fig. 5E
Fig. 5F

F. hCES1

Residual activity (% of control)

Inhibitor concentration (µM)

0 0.01 1 100
Fig. 5G
Supplement data. Effects of inhibitors on MPA formation by human erythrocytes. 450 µL of erythrocytes was preincubated at 37°C for 5 min. Inhibitors were preincubated with erythrocytes at 37 °C for 1 min, and reactions were initiated by the addition of 50 µL MMF solution (final concentration: 100 µM, control activity: 1.56 nmol/min/mL). Each data represents mean of two experiments. BNPP; bis-p-nitrophenylphosphate, DTNB; 5, 5'-ditiobis (2-nitrobenzoic acid).