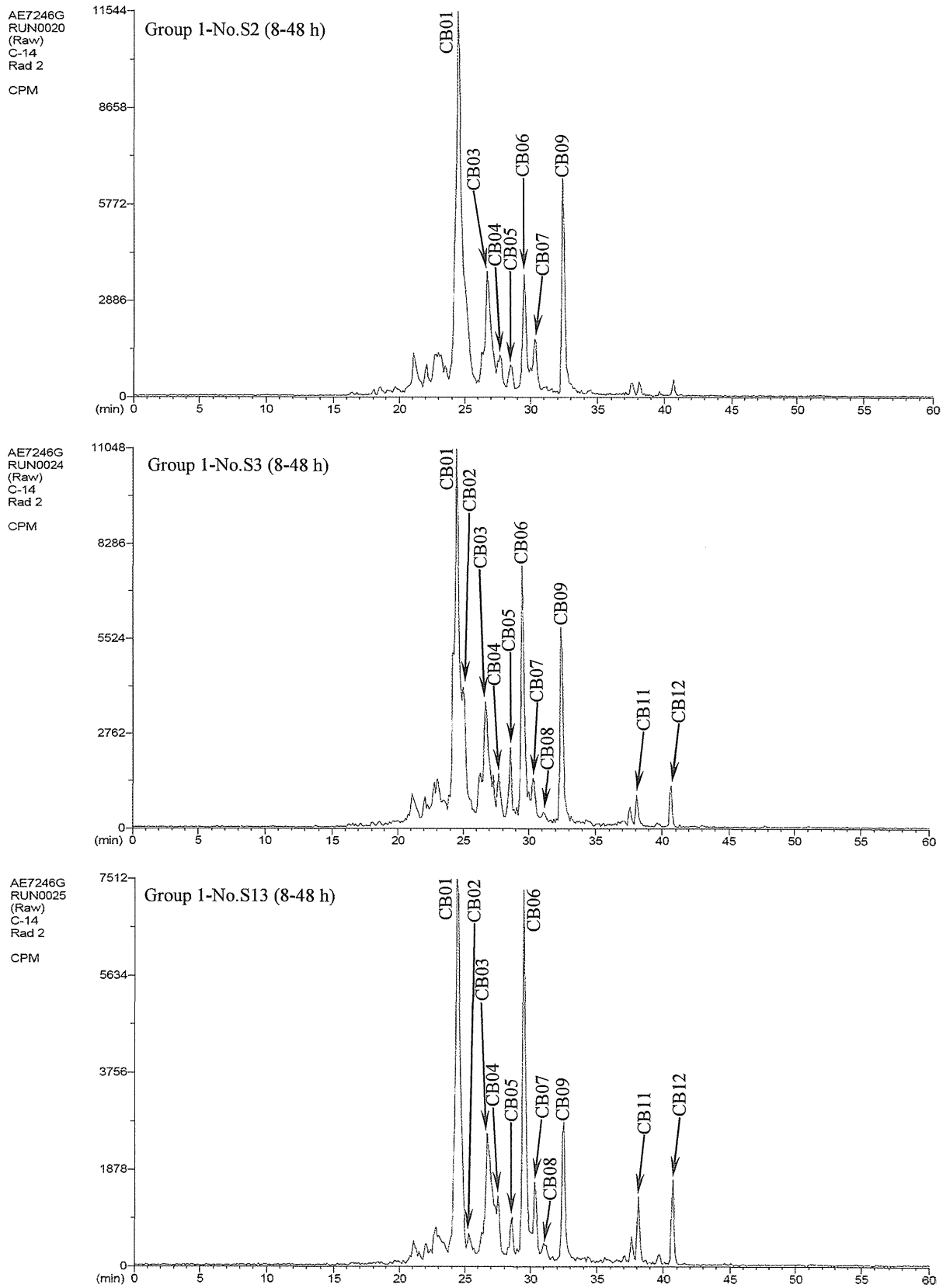

Figure 6 Radiochromatograms of TM5509 and its metabolites in bile after a single oral administration of ^{14}C-TM5509 to fasting male cynomolgus monkeys (dose: 5 mg/kg)

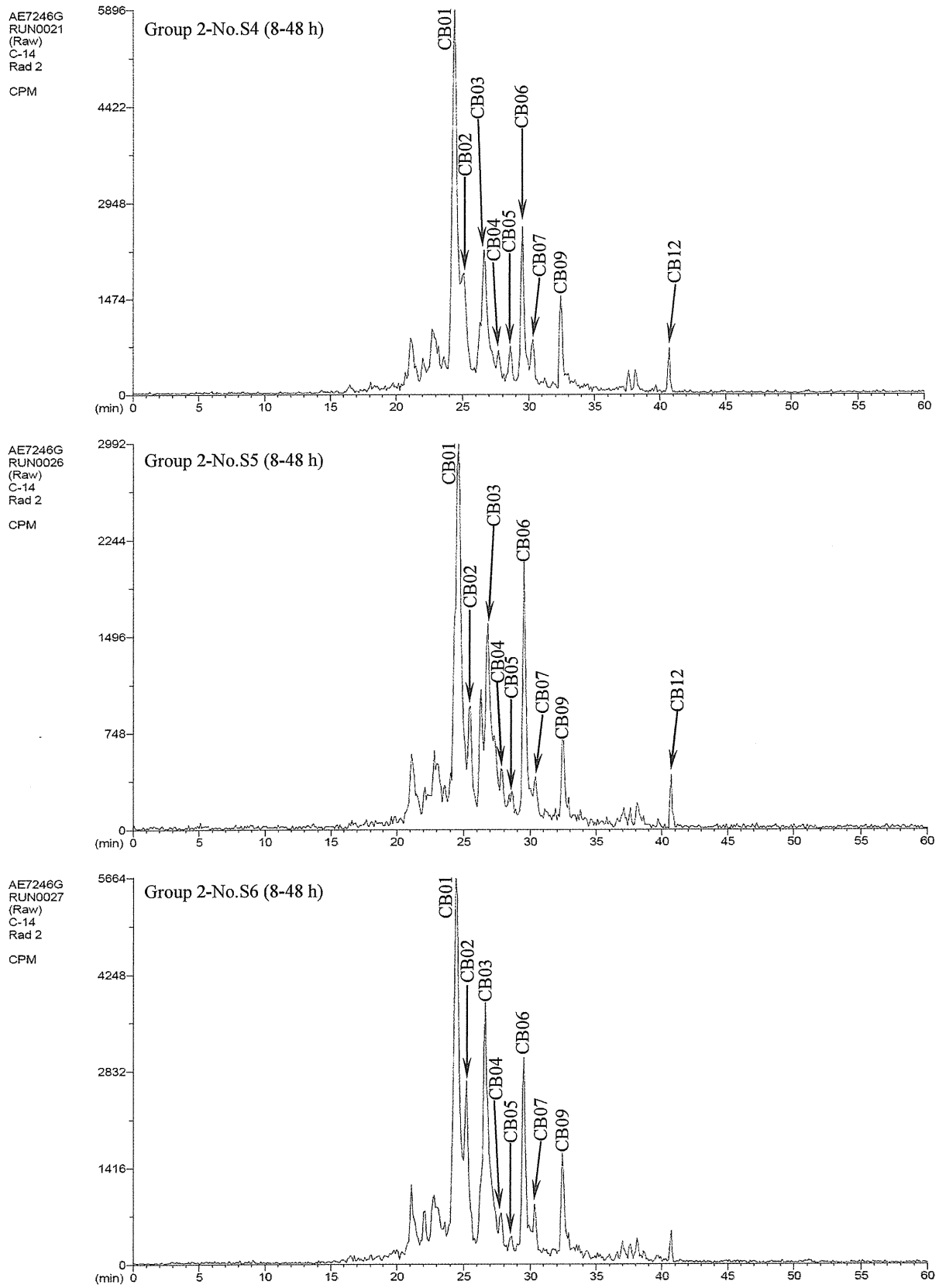

Figure 7 Radiochromatograms of TM5509 and its metabolites in bile after a single oral administration of ^{14}C-TM5509 to fasting male cynomolgus monkeys (dose: 20 mg/kg)

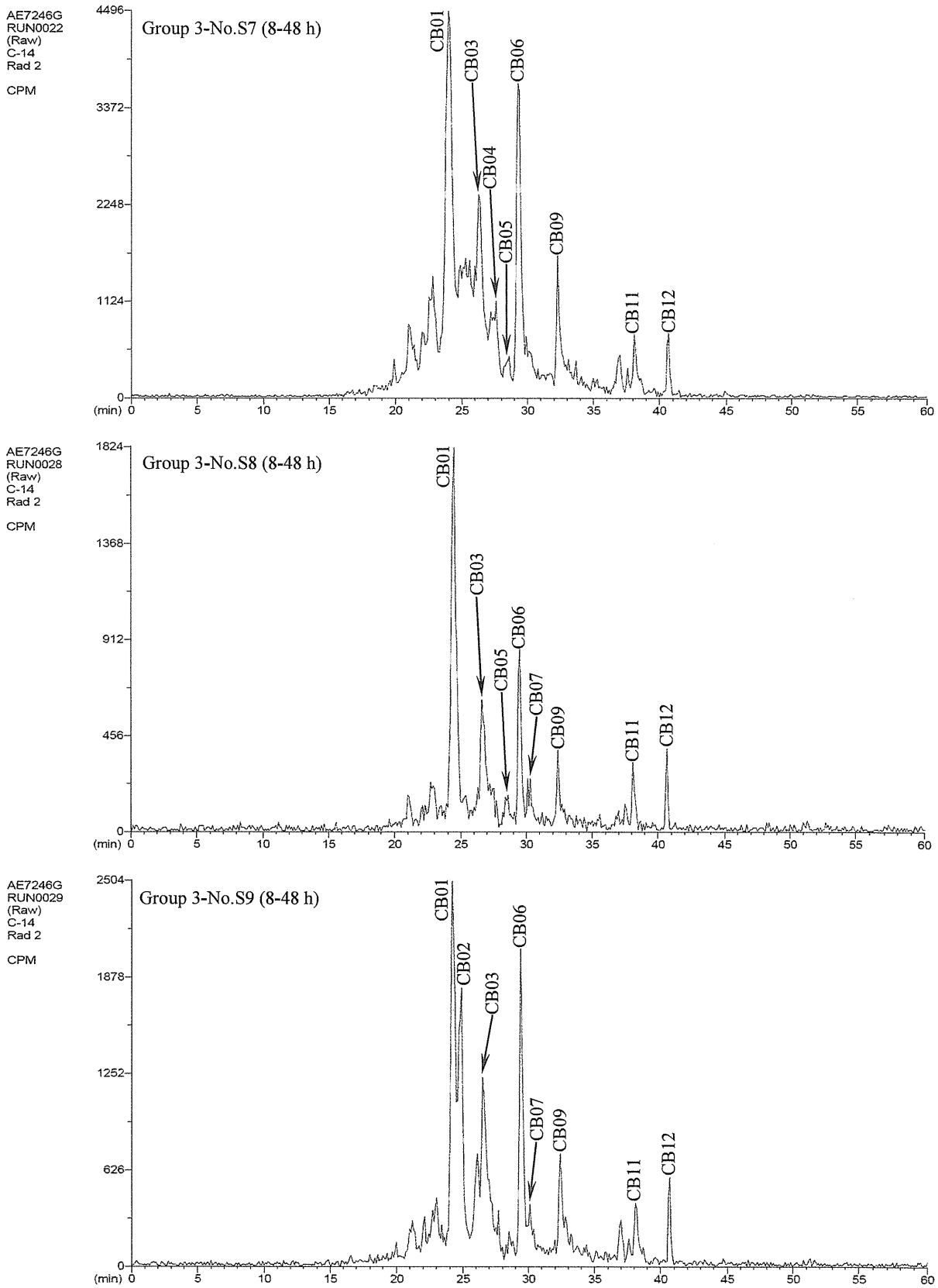

Figure 8 Radiochromatograms of TM5509 and its metabolites in bile after a single oral administration of ^{14}C-TM5509 to fasting male cynomolgus monkeys (dose: 80 mg/kg)

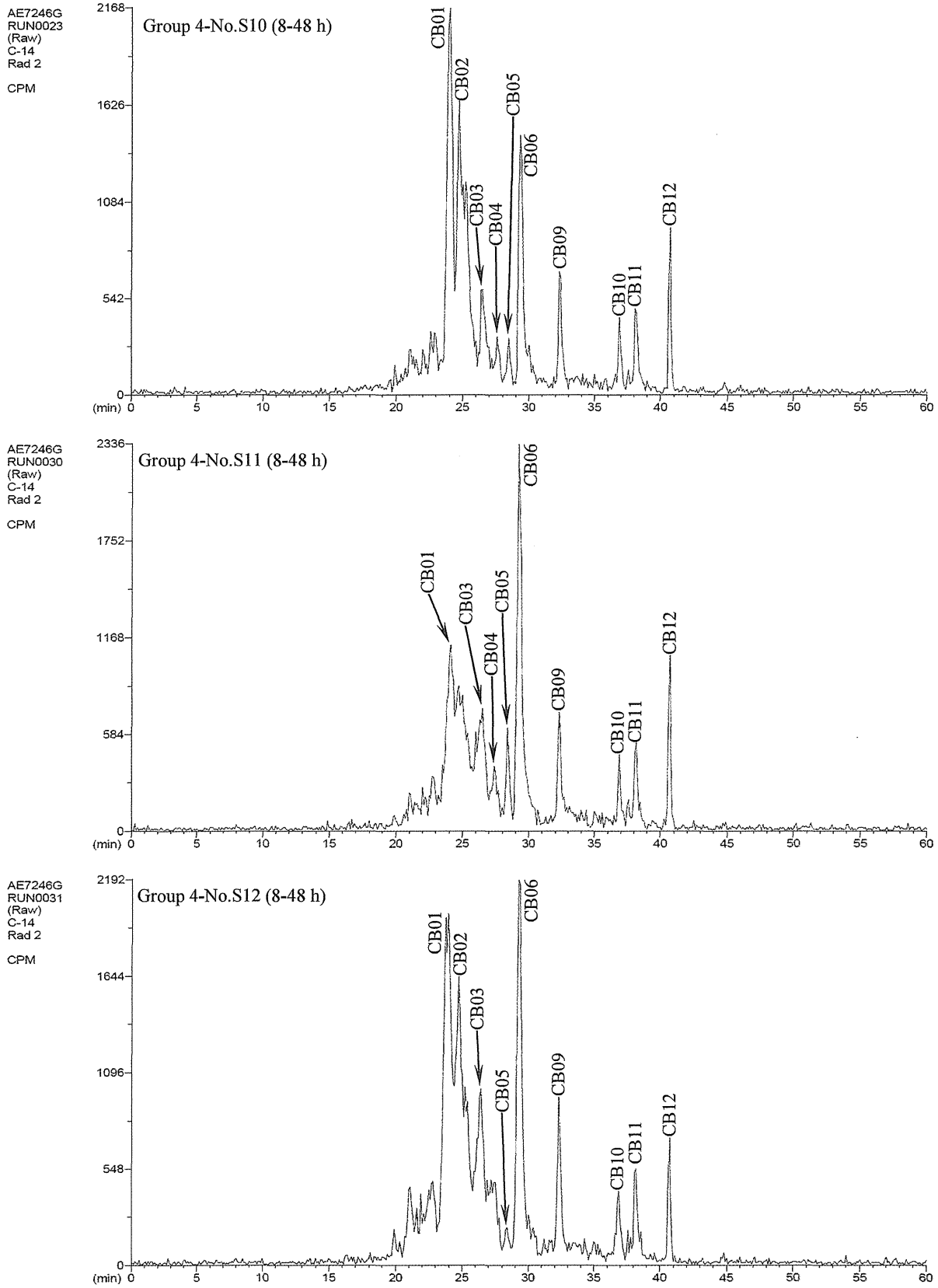

Figure 9 Radiochromatograms of TM5509 and its metabolites in bile after a single oral administration of ^{14}C-TM5509 to fasting male cynomolgus monkeys (dose: 300 mg/kg)

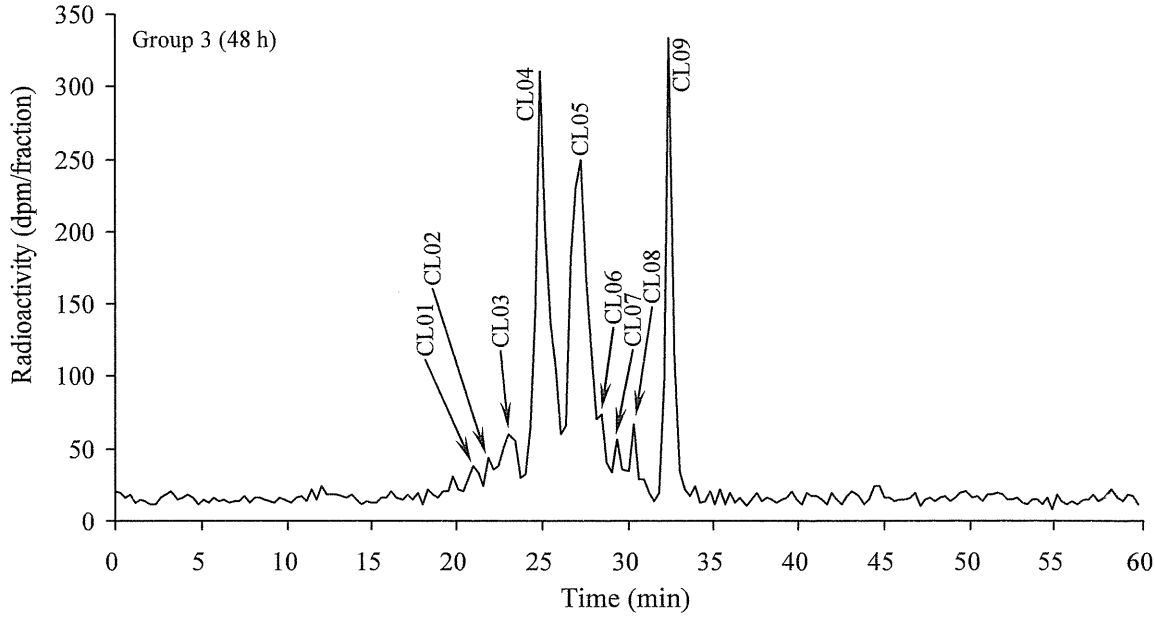

Figure 10 Radiochromatogram of TM5509 and its metabolites in liver after a single oral administration of ^{14}C-TM5509 to fasting male cynomolgus monkeys (dose: 80 mg/kg)

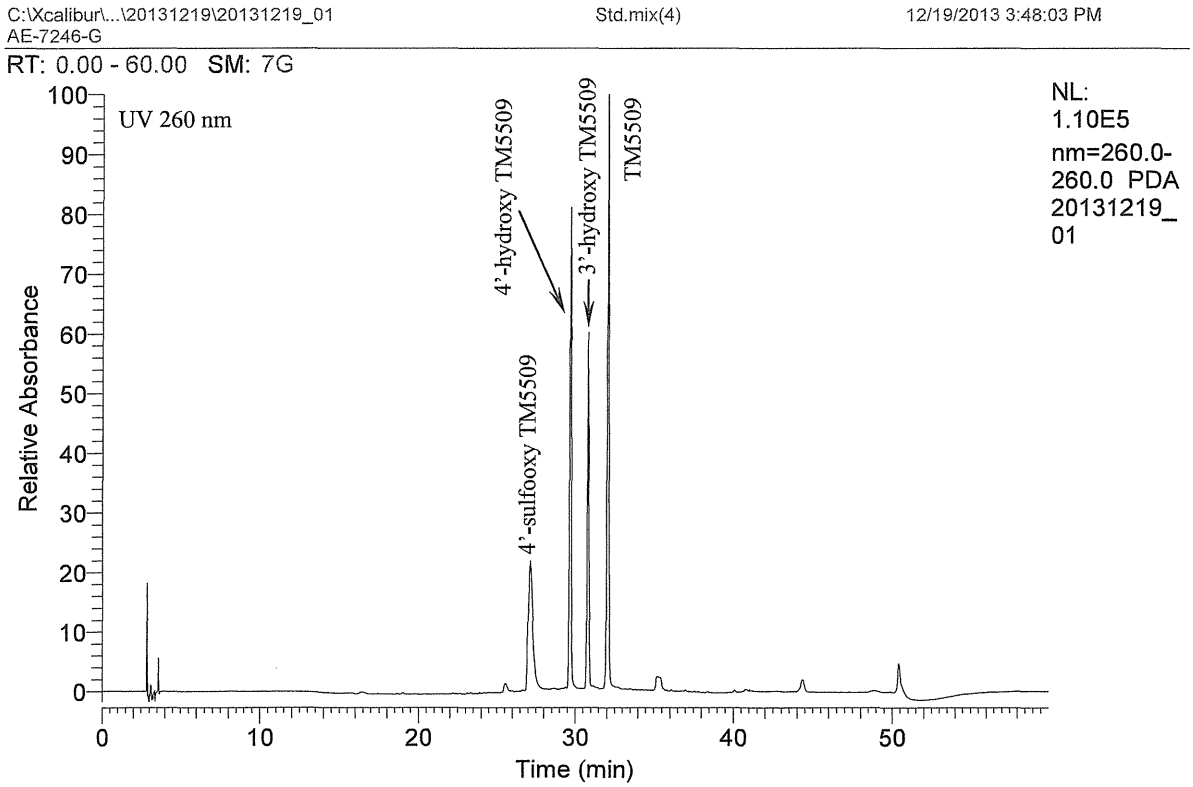

Figure 11 UV chromatogram concurrently acquired with LC-MS^n measurement of authentic standards

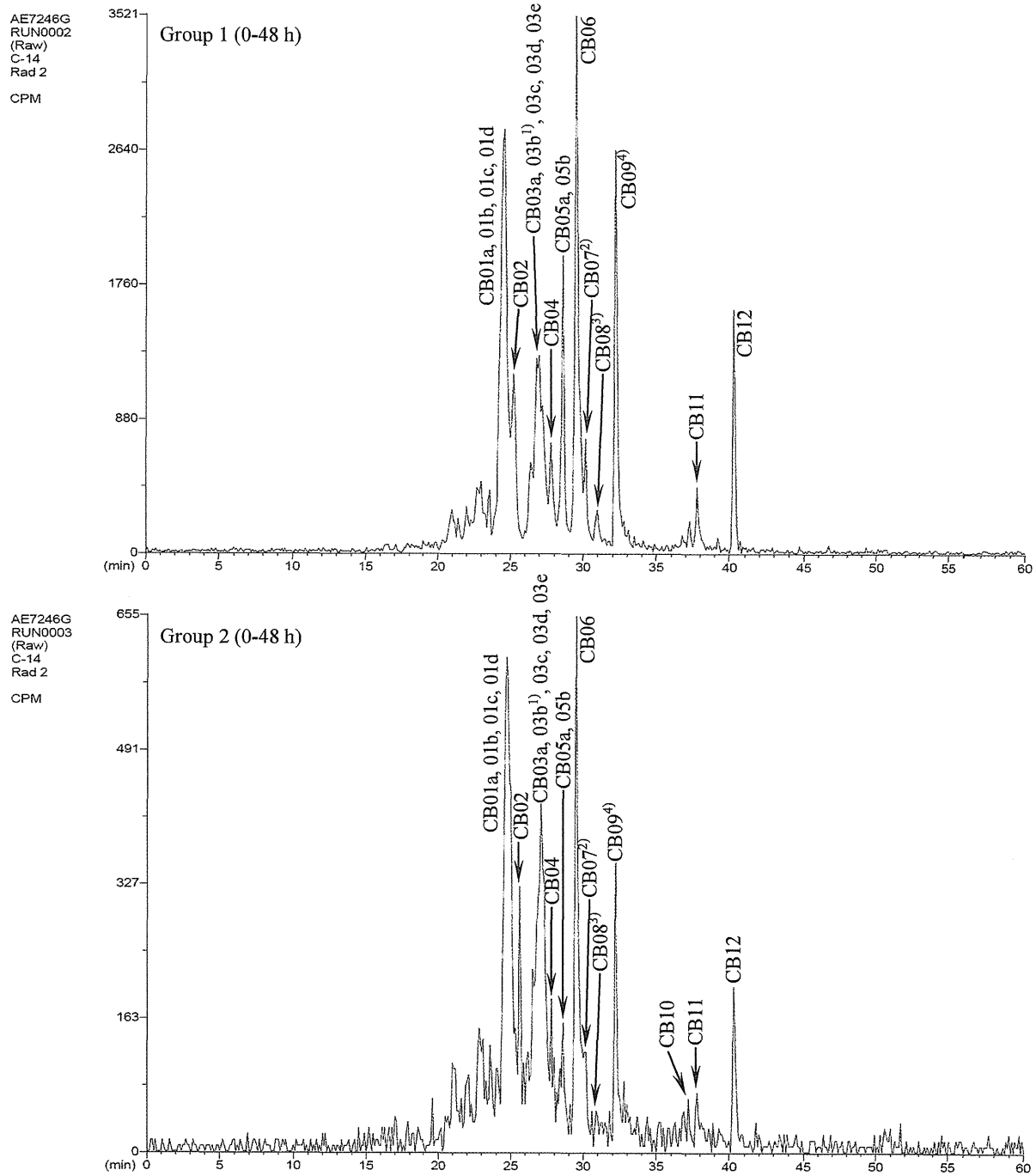

Figure 17 Radiochromatograms of TM5509 and its metabolites in bile after a single oral administration of ^{14}C-TM5509 to fasting male cynomolgus monkeys (continued)

1): 4'-sulfooxy TM5509, 2): 4'-hydroxy TM5509, 3): 3'-hydroxy TM5509, 4): TM5509

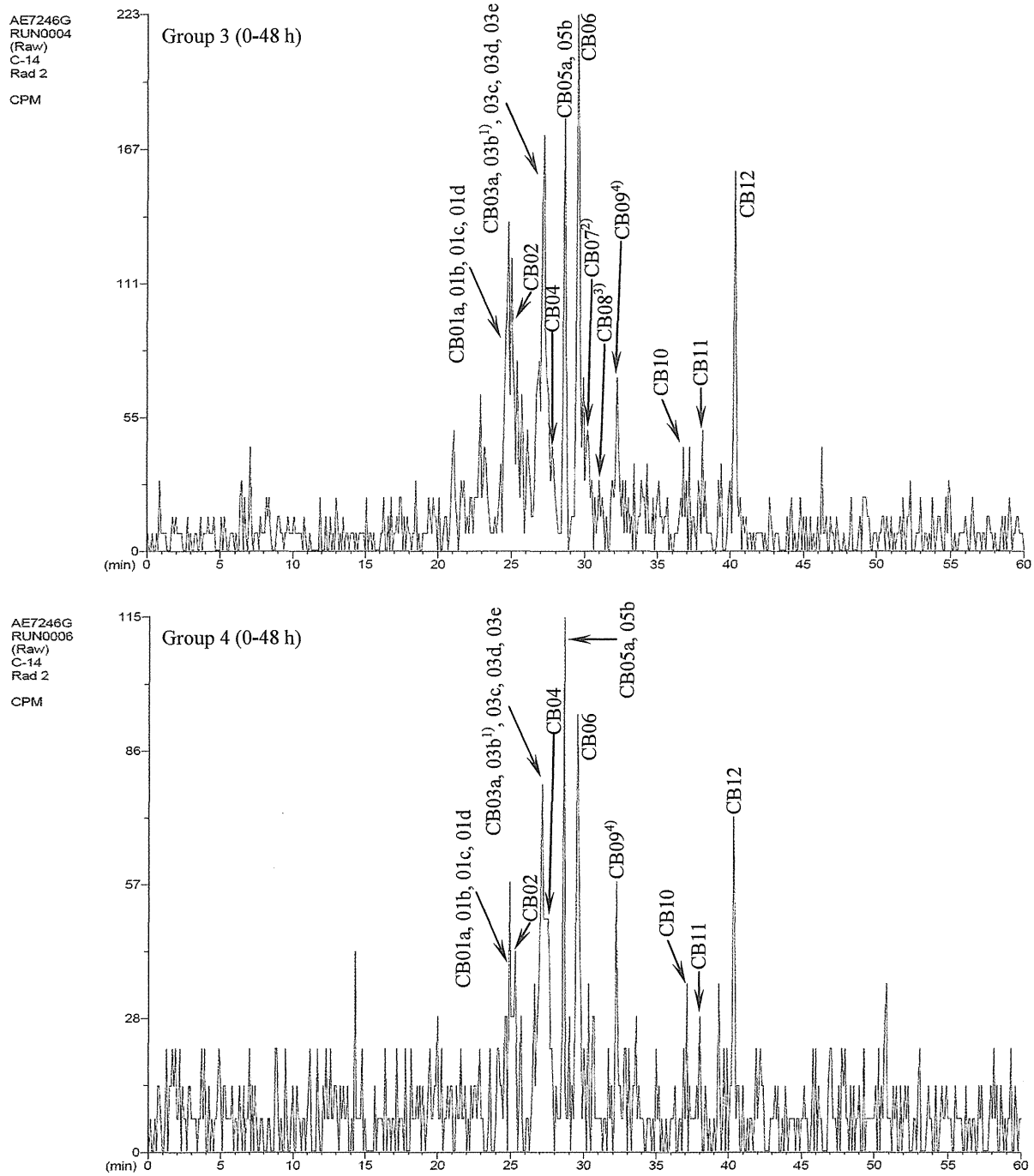

Figure 17 Continued

1): 4'-sulfooxy TM5509, 2): 4'-hydroxy TM5509, 3): 3'-hydroxy TM5509, 4): TM5509

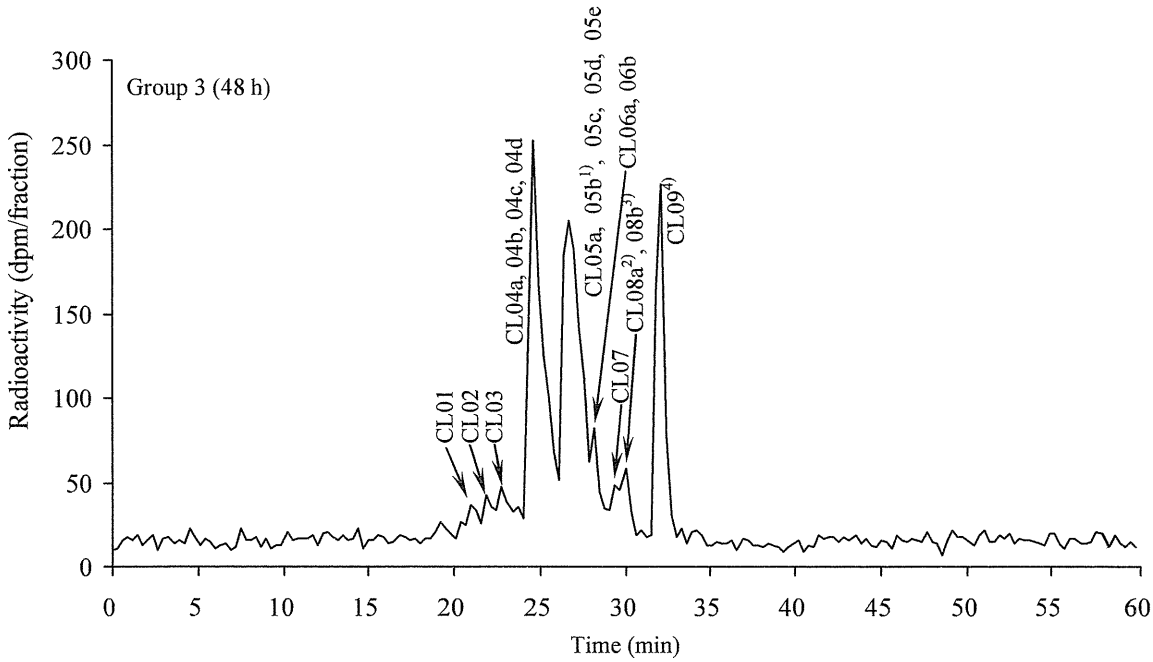

Figure 18 Radiochromatogram of TM5509 and its metabolites in liver after a single oral administration of ^{14}C-TM5509 to fasting male cynomolgus monkeys

1): 4'-sulfooxy TM5509, 2): 4'-hydroxy TM5509, 3): 3'-hydroxy TM5509, 4): TM5509

信頼性保証陳述書

試験表題： ^{14}C-TM5509 の薬物動態試験業務
(^{14}C-TM5509 のサルにおける胆汁中代謝物分析試験)

試験番号： AE-7246-G

上記試験は，薬事法施行規則第 43 条「申請資料の信頼性の基準」に基づいて実施され，また，最終報告書は試験の方法が正確に記載され，かつ生データの内容が正確に反映されていることを確認致しました。信頼性保証部門による調査並びにその結果の報告は，下記の日程で実施致しました。

調査項目	調査者	調査実施日	調査結果報告日*
生データ及び図表（第 1 回目）	片野坂　徳章	2014 年 2 月 27 日～28 日	2014 年 2 月 28 日
生データ及び図表（第 2 回目）	片野坂　徳章	2014 年 3 月 6 日～8 日	2014 年 3 月 8 日
生データ及び図表（第 2 回目：再調査）	片野坂　徳章	2014 年 3 月 10 日	2014 年 3 月 10 日
生データ及び最終報告書草案	片野坂　徳章	2014 年 3 月 11 日～12 日	2014 年 3 月 12 日
生データ及び最終報告書草案（再調査）	片野坂　徳章	2014 年 3 月 13 日	2014 年 3 月 13 日
最終報告書草案	片野坂　徳章	2014 年 3 月 14 日	2014 年 3 月 14 日
最終報告書草案（再調査）	片野坂　徳章	2014 年 3 月 18 日	2014 年 3 月 18 日
最終報告書	樫村　奈央子	2014 年 3 月 26 日	2014 年 3 月 26 日

*: 運営管理者及び試験責任者への報告日

2014 年 3 月 26 日　　積水メディカル株式会社　薬物動態研究所
信頼性保証部門責任者
片野坂 徳章

別添４

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
該当なし							

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Yasui H, Suzuki Y, Brzoska T, Sano H, Suda T, Chida K, Dan T, Miyata T, Urano T.	TM5275 prolongs secreted tissue plasminogen activator retention and enhances fibrinolysis on vascular endothelial cells.	Thromb Res	132	100-105	2013
Remuzzi G, Benigni A, Finkelstein FO, Grunfeld JP, Joly D, Katz I, Liu ZH, Miyata T, Perico N, Rodriguez-Iturbe B, Antiga L, Schaefer F, Schieppati A, Schrier RW, Tonelli M.	Kidney failure: aims for the next 10 years and barriers to success.	Lancet	382	353-362	2013
Miyata T, Suzuki N, van Ypersele de Strihou C.	Diabetic nephropathy: Are there new and potentially promising therapies targeting oxygen biology?	Kidney Int	84	693-702	2013
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Ibrahim AA, Yahata T, Onizuka M, Dan T, van Ypersele de Strihou C, Miyata T, Ando K.	Inhibition of Plasminogen Activator Inhibitor Type-1 Activity Enhances Rapid and Sustainable Hematopoietic Regeneration.	Stem Cells	32	946-958	2014
Miyata T, Ando T, Hiragi H, Watanabe K, Yamamoto F, Vaughan D.E, van Ypersele de Strihou C, Takeuchi M.	Drug discovery and clinical trials in academia.	Nature Review Nephrology	10	290-296	2014

Thrombosis Research 132 (2013) 100–105

Contents lists available at SciVerse ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Regular Article

TM5275 prolongs secreted tissue plasminogen activator retention and enhances fibrinolysis on vascular endothelial cells

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ARTICLE INFO

Article history:
Received 10 January 2013
Received in revised form 5 March 2013
Accepted 1 April 2013
Available online 20 April 2013

Keywords:
fibrinolysis
plasminogen activator inhibitor-1 (PAI-1)
plasminogen activator inhibitor-1 (PAI-1) inhibitor
TM5275
total internal reflection fluorescence (TIRF) microscopy

ABSTRACT

Introduction: Elevated plasminogen activator inhibitor-1 (PAI-1) reduces fibrinolytic potential in plasma, contributing to thrombotic disease. Thus, inhibiting PAI-1 activity is clinically desirable. We recently demonstrated that tissue plasminogen activator (tPA) remains on the surface of vascular endothelial cells (VECs) after secretion in a heavy-chain dependent manner, which is essential for high fibrinolytic activity on the surface of VECs, and that PAI-1 dissociates retained tPA from the cell surface as a result of high-molecular weight complex formation. Based on the model whereby amounts of tPA and its equilibrium with PAI-1 dynamically change after exocytosis, we examined how TM5275, a newly synthesized small molecule PAI-1 inhibitor, modulated tPA retention and VEC surface-derived fibrinolytic activity using microscopic techniques.
Materials and methods: The effects of TM5275 on the kinetics of the secretion and retention of green fluorescent protein (GFP)-tagged tPA (tPA-GFP) on VECs were analyzed using total internal reflection fluorescence microscopy. The effects of TM5275 on the generation of plasmin activity were evaluated by both plasminogen accumulation and fibrin clot lysis on tPA-GFP-expressing VECs using confocal laser scanning microscopy.
Results: TM5275 at concentrations of 20 and 100 μM significantly prolonged the retention of tPA-GFP on VECs by inhibiting tPA-GFP-PAI-1 high-molecular-weight complex formation. TM5275 enhanced the time-dependent accumulation of plasminogen as well as the dissolution of fibrin clots on and around the tPA-GFP-expressing cells.
Conclusions: The profibrinolytic effects of TM5275 were clearly demonstrated by the prolongation of tPA retention and enhancement of plasmin generation on the VEC surface as a result of PAI-1 inhibition.

Introduction

Tissue plasminogen activator (tPA) is a 68-kDa serine protease that initiates fibrinolysis by cleaving a single peptide bond in plasminogen to generate plasmin, which subsequently dissolves fibrin clots in the vasculature. Plasminogen activator inhibitor-1 (PAI-1), a member of the serine protease inhibitor superfamily, regulates both free tPA concentration and tPA activity in plasma by forming a high-molecular-weight complex with tPA [1–5]. Elevated levels of PAI-1 antigen and activity, which are frequently seen in dyslipidemia and metabolic syndrome, naturally reduce fibrinolytic potential in plasma, and contribute to the development of a variety of thrombotic diseases, including deep vein thrombosis, disseminated intravascular coagulation, and coronary artery diseases [6]. The inhibition of PAI-1 activity is thus expected to yield important cardio- and vascular-protective benefits [7], and much attention has been paid to developing inhibitors of PAI-1 [8–10]. One such inhibitor, TM5275, was newly identified by an extensive study of structure-activity relationships based on a lead compound (TM5007) obtained through virtual screening and docking simulations [11,12].

Recently, we successfully visualized the secretory dynamics of green fluorescent protein (GFP)-tagged tPA (tPA-GFP) expressed in vascular endothelial cells (VECs) using total internal reflection fluorescence (TIRF) microscopy, and demonstrated that tPA has unique, slow secretory dynamics and stays on VECs for long periods of time after opening of the secretory granules [13]. This sustained retention of tPA appeared essential for the effective expression of cell surface-associated plasmin activity and the associated fibrinolysis on VECs [14]. We also demonstrated that PAI-1 facilitates the dissociation of tPA from the surface of VECs by forming a high-molecular-weight complex. Thus, PAI-1 appeared to suppress tPA activity not only in plasma but also on the

Abbreviations: tPA, tissue plasminogen activator; PAI-1, plasminogen activator inhibitor-1; VEC, vascular endothelial cell; TIRF, total internal reflection fluorescence; HBS, HEPES-buffered solution; CLSM, confocal laser scanning microscope.

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http://dx.doi.org/10.1016/j.thromres.2013.04.003

surface of VECs. Based on these findings, we hypothesized that TM5275 might critically modulate PAI-1 activity on the VEC surface, where active tPA is constantly exocytosed and its equilibrium with PAI-1 changes dynamically, and thereby potentiate fibrinolytic activity triggered by VEC surface-retained tPA. To test this, we used microscopy techniques to explore the effects of TM5275 on both tPA retention and the expression of fibrinolytic activity on VECs.

Materials and Methods

Cell Culture and Transfection

The human umbilical vein endothelial cell-derived VEC line EA.hy926, which retains endothelial cell-specific functions [15], including fibrinolytic characteristics [16], was kindly provided by Dr C.J. Edgell. We cultured the cells in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St Louis, MO) supplemented with 4.5 g/L glucose and 10% fetal bovine serum at 37 °C under 5% CO_2 in a humidified atmosphere. The cells were cultivated on 35-mm glass bottom dishes (Asahi Techno Glass, Tokyo, Japan) for fluorescence imaging, and on 6-well plates for supernatant analyses. Construction of the tPA-GFP plasmid was described previously [13]. The cells were transfected with the plasmid by lipofection using TransIT-LT1 (Mirus, Madison, WI) at 100% confluence. Experiments were performed within 1 day after transient transfection.

Solutions and Materials

A HEPES-buffered solution (HBS) composed of 140 mM NaCl, 5 mM KCL, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES-NaOH (pH 7.3) supplemented with 3% bovine serum albumin (BSA) (HBS/3%BSA) was used as recording medium, and cells were kept in this solution at 37 °C on the microscope stage (INUG2-ONID-BE; Tokai Hit, Shizuoka, Japan) in all imaging studies. Recombinant tPA (rtPA) was kindly provided by Toyobo-Daiichi (Osaka, Japan), and recombinant PAI-1 (rPAI-1; active, nonglycosylated form) was purchased from Oxford Biomedical Research (Rochester Hills, MI). The ELISA kit for PAI-1 antigen was purchased from Hyphen BioMed (Neuville-sur-Oise, France). Human Glu-plasminogen, purified from fresh-frozen human plasma, and human fibrinogen (Enzyme Research Laboratories, IN) were labeled with Alexa Fluor 568 (Invitrogen, OR) (plg-568) and Alexa Fluor 647 (fbg-647), respectively. Human thrombin was purchased from Benesis (Osaka, Japan). PAI-1 inhibitors, TM5275 [12], synthesized by Hamari Chemicals Ltd (Osaka, Japan), and Tiplaxtinin (Axon Medchem BV, Netherlands) were dissolved in DMSO at a concentration of 0.1% for all studies.

SDS-polyacrylamide Gel Electrophoresis (SDS-PAGE)

The effects of TM5275 on the formation of a tPA-PAI-1 complex were evaluated in a purified system using SDS-PAGE. After incubation with TM5275 at concentrations of 0 (solvent alone), 20, and 100 μM in HBS for 10 min at 37 °C, rPAI-1 (final concentration, 250 nM) was incubated with rtPA (final concentration, 270 nM) for 30 min at 37 °C. After mixing with sample buffer (non-reducing), the mixture was subjected to 10% SDS-PAGE, and the protein bands were stained with Coomassie Brilliant Blue.

Fibrin Autography

To evaluate the effects of TM5275 on the ability of PAI-1 to form high-molecular-weight complexes with tPA either on cultured EA.hy926 cells or in the supernatant, the amounts of tPA-PAI-1 complex and free tPA were semi-quantitated by fibrin autography. Culture media from tPA-GFP-expressing or non-expressing EA.hy926 cells were collected after 3 h of incubation in the presence of 0 (solvent alone), 20, and 100 μM TM5275 at 37 °C, centrifuged at 3,000 ×*g* for 10 min to remove cell debris, mixed with SDS sample buffer, and subjected to 10% SDS-PAGE. tPA-dependent activities were then detected by plasminogen-rich fibrin indicator gels after separation of the protein bands as previously reported [17].

TIRF Microscopic Analysis

We employed a TIRF microscopy unit to evaluate the time that tPA-GFP was retained (tPA-GFP retention time) on cell membrane surfaces after exocytosis from its secretory granules in EA.hy926 cells. This unit enabled us to detect only those fluorophores existing near the plasma membrane facing towards the glass bottom side, as previously described [13]. Briefly, cells were imaged with an inverted microscope (IX81, Olympus, Tokyo, Japan) equipped with a 60X/1.45 numeric aperture oil-immersion objective (for TIRFM, Olympus) to generate the so-called "evanescent field," which illuminates to a depth of ≤100 nm from the glass coverslip into the specimen. We introduced a neutral density filter (Edmund Optics, NJ) to reduce laser intensity to 6%, by which bleaching of intracellular fluorophores was suppressed to less than 10%. GFP was excited at 488 nm, and the emitted light was collected through a 510/23-nm bandpass filter with a 506-nm dichroic mirror. The fluorescence images were captured and recorded every 500 ms through a cooled (−65 °C) high-sensitivity electron multiplier charge-coupled device (EM-CCD; ImagEM, Hamamatsu Photonics, Hamamatsu, Japan) camera controlled by HC Image (Hamamatsu Photonics). We measured the fluorescence intensity of the GFP in each tPA-GFP secretory granule that was exocytosed using the AQUACOSMOS Imaging Station (Hamamatsu Photonics). The "tPA-GFP retention time" $T_{F1/2}$ was defined as the time required for the fluorescence intensity at a single granular spot to decline to one-half of its peak value during the releasing process of tPA-GFP.

Plasminogen-accumulation Analysis

After treatment with 100 μM TM5275 or solvent in HBS/3% BSA for 30 min at 37 °C, tPA-GFP-expressing cells were incubated with human plasminogen (0.5 μM) containing plg-568 (20 nM). The accumulation of plg-568 on or around the cell surface was then analyzed every 10 min with a confocal laser scanning microscope (CLSM; FV1000, Olympus) equipped with a 60X oil-immersion objective lens that captured the fluorescence of plg-568 at wavelengths from 570 nm to 670 nm. Just before the end of each experiment, we added 2.5 μg/mL Cell Mask Deep Red plasma membrane stain (PM; Molecular Probes, Invitrogen, OR) to identify the localization of tPA-GFP-expressing cells. We created a region of interest (ROI) around a single cell, including the pericellular area at the most basal focal plane, and measured the fluorescence intensity using FV10-ASW software (Olympus). Because the mean fluorescence intensity within the ROI increased linearly for 10 min, we calculated the slope of the fluorescence increase over time, representing a time-dependent accumulation of plg-568, and referred to this as dF-plg.

Fibrin Clot Lysis Imaging

tPA-GFP-transfected cells were preincubated with 100 μM TM5275 or solvent in HBS/3%BSA for 30 min at 37 °C. Fibrin clots were then formed over the cells by mixing 0.5 μM human plasminogen, containing 20 nM plg-568, 2 U/mL thrombin, and 1 mg/mL human fibrinogen containing 10 μg/mL fbg-647, in HBS/3%BSA. After fibrin clots were formed on the VECs, we started to collect images every 10 min through an automatically selected dichroic mirror and an appropriate range of

wavelengths for each fluorescent dye using FV1000. We then calculated the lysis area originated from single randomly chosen tPA-GFP expressing cell at a focal plane approximately 3 μm above the bottom of the dish using FV10-ASW software.

Statistical Analysis

All values were analyzed using StatView version 5 (SAS Institute Inc. NC). Normally distributed variables are described as means ± SEM, while non-normally distributed variables (tPA-GFP retention times) are expressed as medians with interquartile ranges. The differences in the amount of plg-accumulation and fibrin clot lysis area were statistically evaluated using Student's *t*-test for paired samples. The Mann–Whitney *U* test was used for the analysis of $T_{F1/2}$. Values of $p < 0.05$ were considered significant.

Results

TM5275 Inhibited High-molecular-weight Complex Formation Between rtPA and rPAI-1 in a Purified System

We first examined the effects of TM5275 on the formation of rtPA-rPAI-1 complexes. In the absence of TM5275, rPAI-1 formed a stable high-molecular-weight complex with rtPA. After treatment with different concentrations of TM5275, the amounts of the high-molecular-weight band with tPA decreased, whilst the amounts of free tPA increased in a dose-dependent manner, indicating that TM5275 effectively reduced the ability of PAI-1 to form a complex with rtPA (Fig. 1). Notably, in the presence of 100 μM TM5275, a small amount of the cleaved fragment of PAI-1 was also detected (Fig. 1, dotted arrow).

TM5275 Prolonged the Retention Time of tPA-GFP on VEC Surface

Based on our recent finding that PAI-1 facilitates the dissociation of tPA from the surface of VECs and shortens the retention time of exocytosed tPA-GFP [13], we used TIRF microscopy to investigate whether TM5275 modifies the release of tPA-GFP from its secretory granules after opening. As previously described [13], the release of tPA-GFP was heterogeneous in each granule, so we calculated the $T_{F1/2}$ values before and 10 min after the addition of 0 (solvent alone), 20 μM, and 100 μM TM5275. Although the frequencies of exocytosis in tPA-GFP secretory granules during each of the 3 min observation periods were similar (12.8 ± 2.3 granules/cell were exocytosed before, and 16.6 ± 3.0 granules/cell after solvent addition; 11.0 ± 2.0 before and 11.2 ± 1.6 after 20 μM TM5275, 17.8 ± 3.1 before and 19.0 ± 0.8 after 100 μM TM5275), the $T_{F1/2}$ was significantly prolonged upon addition of 20 μM and 100 μM TM5275, but was unchanged after solvent alone (Fig. 2). Similarly, another PAI-1 inhibitor, Tiplaxtinin, which reacts with PAI-1 through different epitope, significantly prolonged the retention time {median $T_{F1/2}$; 5.15 seconds (IQR 3.56- 7.34) before (88 granules from 3 experiments) and 6.12 seconds (IQR 4.12- 8.87) after treatment of 100 μM Tiplaxtinin (86 granules from 3 experiments), $p = 0.04$}.

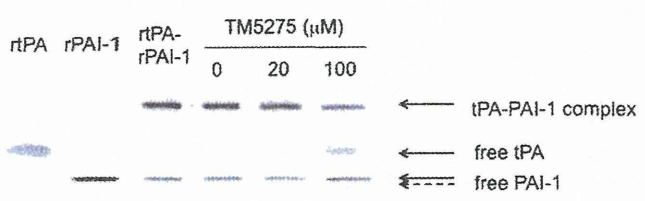

Fig. 1. The inhibitory effect of TM5275 on the formation of complexes between rtPA and rPAI-1. After preincubation with TM5275 at concentrations of 0, 20, 100 μM for 10 min, rPAI-1 was mixed with rtPA for 30 min and the mixture was subjected to SDS-PAGE. Bands corresponding to free, complexed, and cleaved (dashed arrow) forms of PAI-1 are indicated.

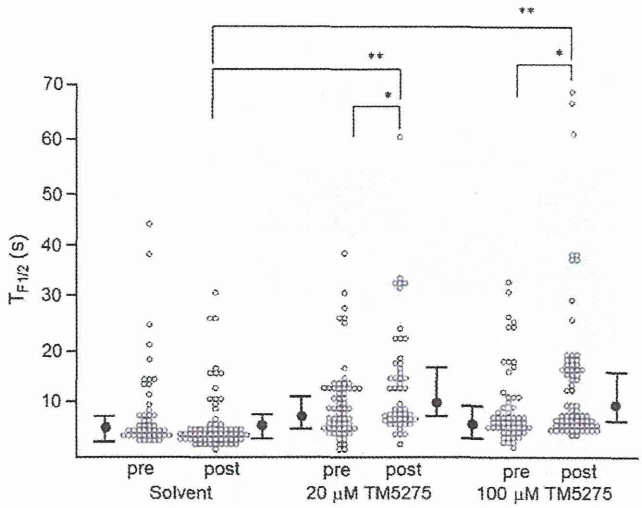

Fig. 2. The Effect of TM5275 on the retention time of tPA-GFP on the surface EA.hy926 cells. Secretory kinetics of tPA-GFP in EA.hy926 cells was analyzed by TIRF microscopy. The distribution of individual $T_{F1/2}$ (tPA-GFP retention time; please see 'Materials and Methods') is shown. $T_{F1/2}$ in 3-min observation periods were calculated (opened circles) before (pre) and after (post) addition of solvent, 20 μM TM5275, 100 μM TM5275 (each 5 independent experiments). Filled circles represent medians and bars the interquartile ranges. *$p < 0.05$, **$p < 0.01$.

TM5275 Inhibited High-molecular-weight Complex Formation Between tPA and PAI-1 on the Surface of EA.hy926 Cells, and Decreased the Amounts of tPA-PAI-1 Complex in Supernatants

Because the prolonged retention of tPA-GFP by TM5275 is most likely due to the inhibition of high-molecular-weight complex formation between tPA-GFP and PAI-1 on the surface of tPA-GFP-transfected EA.hy926 cells, we analyzed the amounts of tPA-PAI-1 and tPA-GFP-PAI-1 complex in the supernatants of transfected and non-transfected EA.hy926 cells after TM5275 treatment using plasminogen-rich fibrin indicator gels. The strength of plasminogen activation-dependent lysis of the high-molecular-weight band of endogenous tPA-PAI-1 complex was weaker after treatment with 100 μM TM5275 (Fig. 3A). Similarly, the lytic intensities of the high-molecular-weight bands of both the tPA-GFP-PAI-1 complex and the endogenous tPA-PAI-1 complex in tPA-GFP-expressing cells were weaker after treatment with TM5275 in a dose dependent manner (Fig. 3A). These results indicate that TM5275 successfully inhibited VEC-derived PAI-1 activity and suppressed high-molecular-weight complex formation with both endogenous- and GFP-conjugated- tPA.

To confirm that TM5275 did not modify PAI-1 expression in EA.hy926 cells, the concentrations of total PAI-1 in the culture media were determined by ELISA. The concentrations of total PAI-1 in the presence of 0 (solvent alone), 20 μM, and 100 μM TM5275 were similar (36.7 ± 3.2, 38.2 ± 7.3, and 36.1 ± 2.8 ng/ml, respectively; Fig. 3B). Furthermore, the free form of tPA did not appear in the supernatant, even after treatment with 100 μM TM5275 (Fig. 3A). Larger amounts of both endogenous- and GFP-conjugated tPA seem to have remained on the surface of EA.hy926 cells, possibly as a result of attenuated PAI-1 activity.

Effect of TM5275 on Accumulation of Plasminogen on and Around the VECs

We previously showed that sustained retention of enzymatically active tPA on the surface of VECs effectively enhanced plasmin activity and further enhanced the accumulation of plasminogen on and

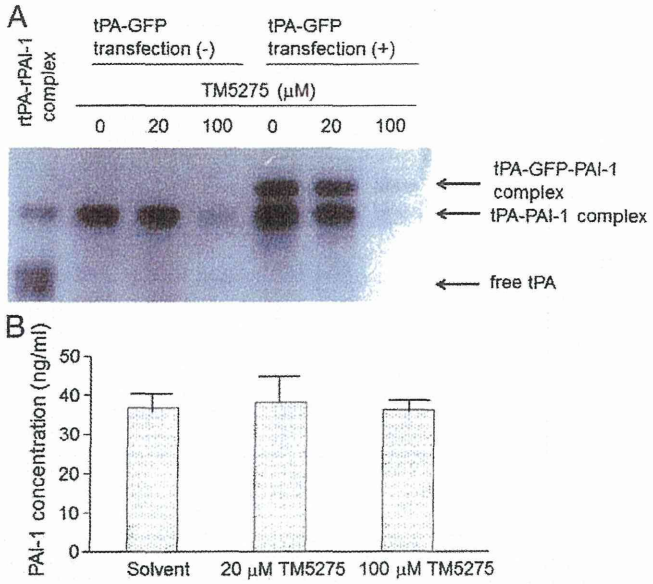

Fig. 3. The inhibitory effect of TM5275 on cell-derived PAI-1 activity in EA.hy926 cells. **A**, Three-hour culture media treated with or without TM5275 from untransfected or tPA-GFP-transfected cells were analyzed by plasminogen-rich fibrin autography. **B**, The concentrations of total PAI-1 measured by ELISA in the same samples from untransfected cells in panel A (3 independent experiments) are shown as mean ± SEM. There was no significant difference.

around the cells [14]. As this enhanced accumulation of plasminogen was lysine-binding site-dependent and required enzymatic activities of both tPA and plasmin, the underlying mechanism was suggested to be proteolytic cleavage of cell surface associated proteins by generated plasmin and the exposure of newly generated C-terminal lysine [14]. To examine this, we evaluated the effect of the PAI-1 inhibitor TM5275 on cell surface-associated plasminogen accumulation, by measuring the fluorescence intensity of plg-568 (dF-plg) on tPA-GFP-expressing EA.hy926 cells using CLSM. Similar to our previous study [14], the red fluorescence of plg-568 appeared not only at the plasma membrane but also at the pericellular/matrix adhesive area (Fig. 4A, B). The fluorescence intensity of plg-568 increased with time after treatment with TM5275, and was markedly stronger than that following the addition of solvent alone (Fig. 4A, B), although the fluorescence intensities of tPA-GFP were similar (green intensities/cell; 117.4 ± 18.3 in solvent and 101.2 ± 24.0 in TM5275 from 18 cells and 20 cells, respectively; mean ± SEM in 6 independent experiments). Relative dF-plg was significantly higher in TM5275-treated cells (18 cells from 6 independent experiments) compared to solvent-treated cells (20 cells from 6 independent experiments) (Fig. 4C). These results suggest that TM5275 effectively inhibited PAI-1 and enhanced tPA activity on the surface of EA.hy926 cells, which resulted in enhanced cell surface-associated plasminogen accumulation.

Efficacy of TM5275 on Lysis of Fibrin Clot Overlaid on VECs

Finally, we evaluated whether TM5275 enhances the cell surface-associated dissolution of fibrin clots that formed on the tPA-GFP-expressing cells. After the formation of a fibrin network, the filamentous structure and its lysis were clearly demonstrated by sequential monitoring via CLSM (Fig. 5A, B). The localized, linear accumulation of plg-568 at the lytic edge of the fibrin structure was always observed along with the expansion of the lytic zone as described before [14], which made it easier to measure the lysis area. Lysis areas after TM5275 treatment were significantly larger than those after solvent treatment at 30 min and 40 min in every 10-min analysis (Fig. 5C). Inhibition of PAI-1 activity by TM5275 appeared to potentiate VEC-associated fibrinolytic activity on the cell surface.

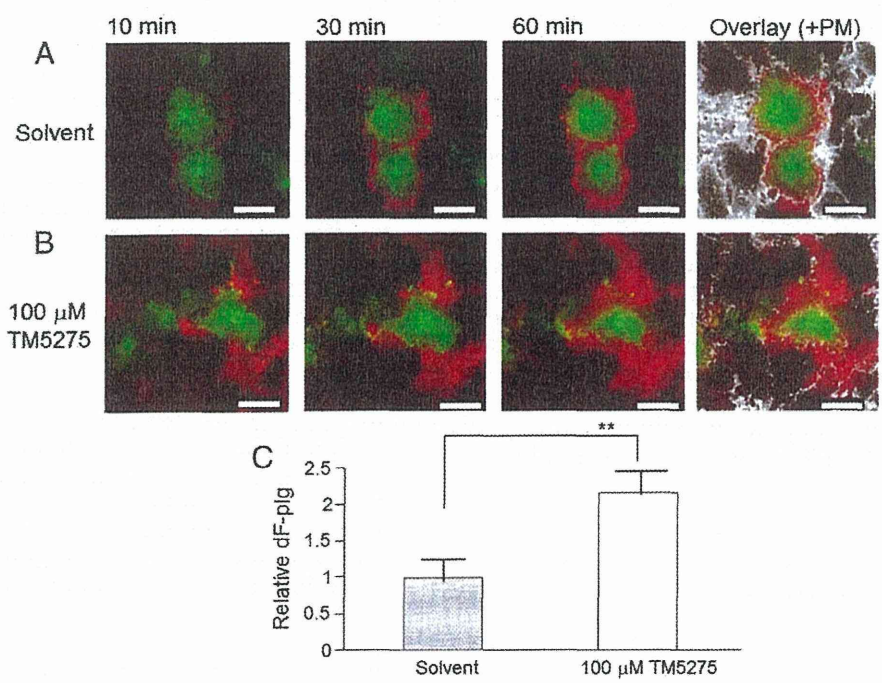

Fig. 4. Effect of TM5275 on accumulation of plasminogen on and around tPA-GFP-expressing EA.hy926 cells. Representative images of tPA-GFP (green) and plg-568 (red) captured by CLSM are shown at indicated times (10, 30, and 60 min) after addition of plg-568 in tPA-GFP-expressing cells, which were preincubated with solvent (**A**) or 100 μM TM5275 (**B**) for 30 min. Plasma membranes stained with Cell Mask Deep Red plasma membrane stain (white) after capturing the images at 60 min are shown in the 'overlay' right panels. Scale bars represent 20 μm. **C**, The amounts of dF-plg, calculated from the slope of the plg-568 fluorescence increase per time and normalized to that of the solvent, are shown as mean ± SEM from 6 independent experiments. **$p < 0.01$.

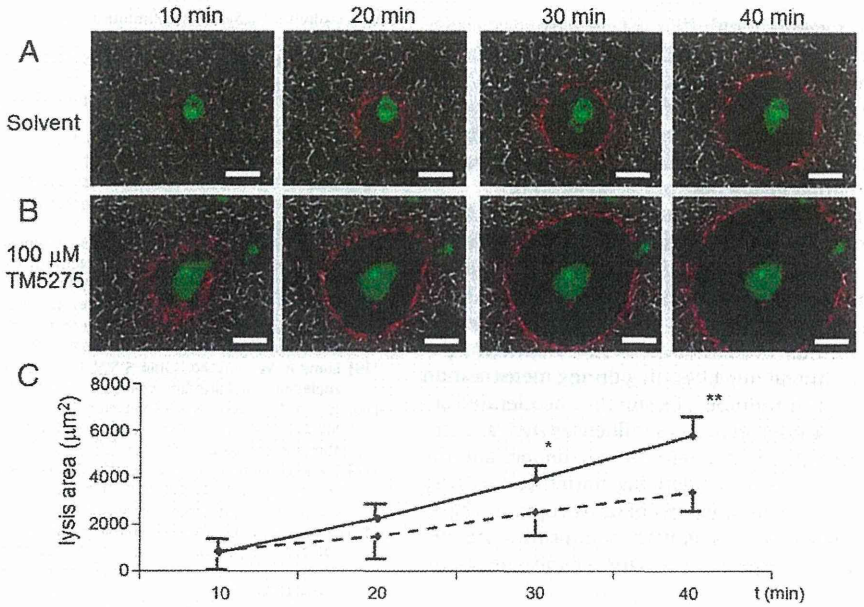

Fig. 5. Effect of TM5275 on lysis of fibrin clot overlaid on tPA-GFP-expressing EA.hy926 cells. Representative images during fibrin clot lysis on tPA-GFP-expressing cells at a focal plane approximately 3 μm above the bottom of the culture dish at indicated times (10, 20, 30, 40 min) after fibrin clot formation on cells pretreated with solvent (**A**) or 100 μM TM5275 (**B**). Green: tPA-GFP, red: plg-568, white: fbg-647. Scale bars represent 20 μm. **C**, Lysis area at the same focal plane as shown in panels A and B were calculated every 10 min. Results of 10 independent experiments pretreated with solvent (dashed line) or 100 μM TM5275 (solid line) are shown as mean ± SEM. *$p < 0.05$, **$p < 0.01$ versus solvent.

Discussion

In this study, we directly demonstrated that TM5275, a small molecule PAI-1 inhibitor, successfully enhanced both tPA- and fibrinolytic activity on VECs as a result of PAI-1 inhibition. Although the therapeutic efficacy of this compound was demonstrated in the monkey photochemically induced thrombosis model by showing the prolongation of occlusion time without changing the bleeding time [12], the direct evidence of this compound's ability to enhance fibrinolysis by inactivating PAI-1 had not been demonstrated in previous *in vitro* experiments, conducted mostly under static conditions. Our recently established method [13,14] to analyze tPA-secretory dynamics and the associated fibrinolytic activity, in which the amounts of tPA and its equilibrium with PAI-1 dynamically change due to exocytosis, enabled us to analyze the direct effects of TM5275 on both the interaction between just excytosed tPA and PAI-1 and the associated fibrinolytic activity.

PAI-1, a primary regulator of fibrinolysis, contains no disulfide bonds, which makes it easy to alter its conformation to the active-, latent- and substrate-forms [18,19]. This vulnerability of PAI-1 to conformational changes and its activity were targeted in developing an inhibitory molecule to reduce PAI-1 activity. To date, several PAI-1 inhibitors have been developed and characterized. TM5275, having been developed as a small molecular PAI-1 inhibitor, is simulated to dock to strand 4 of the A β-sheet (s4A) of the PAI-1 molecule [12], and then expected to convert active PAI-1 to its inactive forms. One possibility of this characteristic was documented in the present study by clearly showing the inhibition of PAI-1 to form a high-molecular-weight complex with tPA and its cleavage to smaller fragments, which indicated that TM5275 converted active PAI-1 to its substrate form to some extent (Fig. 1, dotted arrow).

We previously [13] clarified the inhibitory role of PAI-1 in retaining tPA on cell surfaces using three different approaches: (1) in cell culture experiments, supplementing the culture media with rPAI-1 for 60 min shortened the $T_{F1/2}$ of tPA-GFP and increased the amount of intrinsic tPA-rPAI-1 complex, (2) a GFP-tagged mutant form of tPA, tPA-S478A-GFP, in which Ser478 was substituted by Ala so as to be catalytically inactive and unable to form covalent complexes with PAI-1, exhibited a prolonged $T_{F1/2}$ and its complexed form with PAI-1 (tPA-S478A-GFP-PAI-1) was not detected in the media, and (3) suppression of PAI-1 synthesis by siRNA prolonged the $T_{F1/2}$ of tPA. We concluded, therefore, that PAI-1 decreases the amounts of cell surface-associated tPA on VECs by facilitating the dissociation of tPA from the cell surface.

In agreement with these findings, TM5275 suppressed the PAI-1-dependent dissociation of tPA from the cell surface and prolonged the $T_{F1/2}$ of tPA-GFP (Fig. 2), which was demonstrated by decreased amounts of both the tPA-GFP-PAI-1 complex and the endogenous tPA-PAI-1 complex in fibrin autography (Fig. 3A), even though the total amounts of PAI-1 in the supernatant were similar. As free tPA was not detected in the supernatants after TM5275 treatment, exocytosed tPA was assumed to stay on the cell surface in an active form. The retention of larger amounts of active tPA after TM5275 treatment was confirmed by our findings that TM5275 enhanced pericellular plasminogen accumulation and subsequent cell surface-dependent fibrinolysis. Thus, TM5275 appeared to enhance both the activity of endogenous tPA and fibrinolysis by inhibiting PAI-1 on the surface of VECs, where active tPA is constantly exocytosed and the equilibrium between tPA and PAI-1 is dynamically changed.

An orally active PAI-1 inhibitor holds great therapeutic promise for a variety of clinical indications. Thrombotic complications of vascular disease constitute the leading cause of morbidity and mortality in much of the developed world. Patients most likely to benefit from PAI-1 inhibition would be those at high risk for vascular events where PAI-1 is elevated, as is observed in obesity, diabetes, and the metabolic syndrome. The first orally active small molecule PAI-1 inhibitor, Tiplaxtinin has been demonstrated to prevent thrombus formation after vascular injury in rat models [20], and it was also demonstrated to effectively prolong the retention of exocytosed tPA-GFP in our experimental model. Tiplaxtinin is invalid, however, for vitronectin-bound PAI-1 because this compound binds to the vitronectin binding domain in PAI-1 molecule [21]. Since TM5275 recognizes another epitope of s4A of PAI-1 molecule, further beneficial effects would be expected in the prevention of thrombosis under those pathological conditions by effectively enhancing endogenous fibrinolytic activity.

Furthermore, TM5275 is expected to enhance the thrombolytic effect of intravenous administrated rtPA. Combinatory use of small

molecule PAI-1 inhibitors together with rtPA may decrease the therapeutic dosage of rtPA and its associated complications, including symptomatic intracranial hemorrhage [22] and neurotoxic events. The usefulness of the combination therapy of rtPA and TM5275 was demonstrated in the rat thrombosis model, whereby it successfully prolonged occlusion time without prolonging bleeding time, which was comparable with the effect of a 10 times higher dose of rtPA alone [12].

In contrast to other thrombolytic reagents, including rtPA, PAI-1 inhibitors are believed to not cause serious bleeding. This is based on the fact that PAI-1-deficient mice showed no significant abnormalities in bleeding tests [23,24]. Caution should be exercised, however, because the phenotype of the PAI-1-deficient mice is completely different from that of homologous PAI-1-deficient patients, who exhibited life-threatening bleeding during menstruation and surgical operations [25]. In addition, because the accelerated activity of tPA is reported to cause deleterious influences such as damage of blood brain barrier [26], it is urgent that we understand the details of the regulatory mechanisms defining fibrinolytic activity on cell surfaces as well as the consequences of its disruption. Therefore, as in other anti-coagulants, an adequate monitoring system of the remaining PAI-1 activity appears necessary for the safe use of an oral PAI-1 inhibitor.

In conclusion, this study demonstrated using microscopic techniques that TM5275, a novel PAI-1 inhibitor, enhanced fibrinolytic activity on VECs. We believe that this model, whereby tPA and PAI-1 are in dynamic equilibrium, is advantageous for the evaluation of the efficacy of new pharmacological PAI-1 inhibitors to facilitate thrombus resolution in human *in vitro* systems.

Conflict of Interest Statement

None

Author's Contribution

Hideki Yasui: Conception and design, Data collection, Data analysis and interpretation, Manuscript writing; Yuko Suzuki: Conception and design, Data analysis and interpretation, Manuscript writing; Hideto Sano, Takafumi Suda, Kingo Chida, Dan Takshi and Toshio Miyata: Data analysis and interpretation; Tetsumei Urano: Conception and design, Data analysis and interpretation, Final approve of manuscript. All authors read and approved the final manuscript.

Acknowledgements

This work was supported by Grants-in-Aid for Scientific Research (C:22590826 [Y.S.] and C:24590273 [T.U.]) from the Japan Society for the Promotion of Science, and a grant from the Smoking Research Foundation (T.U.).

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Global Kidney Disease 6

Kidney failure: aims for the next 10 years and barriers to success

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Although in some parts of the world acute and chronic kidney diseases are preventable or treatable disorders, in many other regions these diseases are left without any care. The nephrology community needs to commit itself to reduction of this divide between high-income and low-income regions. Moreover, new and exciting developments in fields such as pharmacology, genetic, or bioengineering, can give a boost, in the next decade, to a new era of diagnosis and treatment of kidney diseases, which should be made available to more patients.

Lancet 2013; 382: 353–62

Published Online May 31, 2013 http://dx.doi.org/10.1016/S0140-6736(13)60438-9

This is the sixth in a Series of six papers about global kidney disease

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Introduction

Acute kidney injury and chronic kidney diseases are substantial health concerns. Acute kidney injury is still associated with high mortality, whereas chronic kidney disease is directly, or as a risk factor for cardiovascular disease, an economic burden to health systems.

Strategies are now available to slow down or stop the progression of chronic kidney disease, but poverty has prevented their application to most patients worldwide. Moreover, in low-income countries, several disorders that lead to acute kidney injury are potentially preventable or avoidable (panel 1).

Acute kidney injury: a preventable and treatable disorder

In low-income countries, acute kidney injury is usually associated with infections, nephrotoxins, or obstetric and surgical complications.[1–4] Limited resources for diagnosis, late or no referral to nephrology services, or lack of access to renal replacement therapy are key challenges. Renal replacement therapy is usually only obtainable in large cities for those who can afford to pay.

The challenge for the international nephrology community is how to support the development of strategies in low-income countries that allow timely diagnosis of acute kidney injury and provide access to renal replacement therapy for patients with potentially reversible disease.

Although highly specialised dialysis techniques are not feasible in low-income countries, peritoneal dialysis is affordable[5–7] and appropriate in environments with limited technology. However, sustainability of peritoneal dialysis can only be achieved with full local commitment and availability of necessary professional skills. A peritoneal dialysis programme for acute kidney injury that does not need electricity and machines can be started even in extremely low-technology environments. Compared with a haemodialysis programme, peritoneal dialysis is less costly to set up and maintain, although it can be sustainable only when governments include them in the national health-care budget. One important challenge is in being able to buy equipment for peritoneal dialysis.

Supporting the development of such programmes with educational grants for training and provision of start-up funds are appropriate objectives for the international community. Development of sustainable models from a resource and financial viewpoint is a bigger challenge, but is the ultimate objective; meeting this challenge will require co-operation and commitment of local health-care facilities, hospitals, and governments.

The Kilimanjaro Christian Medical Centre in Moshi, Tanzania, has developed a feasible programme for peritoneal dialysis in acute kidney injury, an initiative coordinated by the Sustainable Kidney Care Foundation in New York, and three universities in the USA and Canada, with the support of the International Society of Nephrology and the country's Ministry of Health.[8]

In summary, many patients with acute kidney injury in low-income countries will recover kidney function and

Search strategy and selection criteria

References included in this Series were identified by the authors, based on their respective areas of expertise and supplemented by unsystematic database searches.

Key messages

The goals for nephrology in the next decade are:

- To reduce the burden of preventable causes of acute kidney injury in low-income countries and promote affordable renal replacement therapies
- To make worldwide interventions available that help combat the burden of chronic kidney diseases with selective screening, infant and maternal health care, and prevention and treatment of curable diseases
- To develop new drugs for kidney diseases
- To create new methods for diagnosis and treatments for inherited kidney disease
- To develop and apply bioengineering sciences to repair damaged tissues, and generate new organs

Panel 1: Case report

A girl aged 15 years in Tanzania developed acute kidney failure with oliguria. Her recent medical history and her clinical and laboratory findings were compatible with more than one cause of acute kidney failure, including haemolysis, ischaemia associated with subacute bacterial infection, ischaemic sickle cell disease, and postinfectious glomerulonephritis. The availability of a small peritoneal dialysis programme at the hospital allowed the girl to have renal replacement therapy to treat acute kidney failure. After 14 days of treatment, her kidney function completely recovered. She was discharged and continued to attend school, receiving hydroxycarbamide for sickle cell disease.

Panel 2: Steps to support and develop programmes of prevention and treatment of acute kidney injury in low-income countries

- Agreement by the physicians, hospital administration, and nursing staff on a regional level that treatment programmes for acute kidney injury be developed
- Assessment of available resources: medical and financial
- Development of programmes to diagnose acute kidney injury in a timely fashion at local health-care facilities
- Determination of the optimum renal replacement therapy to be provided
- Development of a viable financial model to support the programme
- Provision of training for appropriate individuals (physicians, nurses, other health-care providers, and administrators)
- On-going support by the international community; for example in terms of consultations and resource re-evaluation
- Involvement of the international community to assist in the planning, development, and implementation of these programmes

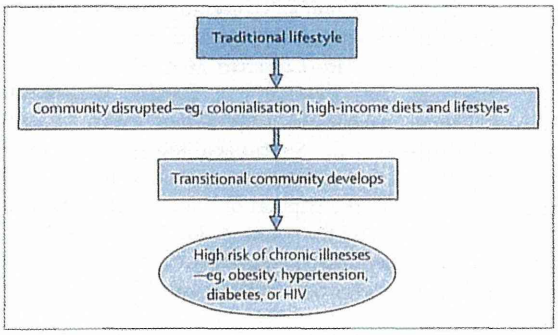

Figure: Transitional communities and the risk of chronic diseases including chronic kidney disease

survive if appropriate renal replacement therapy is provided. In the next decade the international community should support initiatives with the goal that nearly all patients with potentially reversible acute kidney injury should be offered appropriate supportive care, including dialysis, at a sustainable cost. Steps that need to be taken are summarised in panel 2. Saving Young Lives in Africa and Asia (2012–16), is one such programme for prevention and treatment of acute kidney injury.[9]

To achieve the goal of universal access to treatment, it is important that the international community is involved to ensure successful development of these programmes, and innovative partnerships among the private sectors, foundations, academic institutions, and governments are incumbent.

Maternal and infant health promotion

In high-income countries, women are at the same risk of chronic kidney disease as men.[10] However, unique to women is the development of pregnancy-associated kidney diseases and their sequelae. These challenges are magnified in developing countries.[11,12] Another paper in this Series[13] has alerted us to the importance of maternal diseases and early fetal and childhood development as a precursor of chronic non-communicable diseases. The cycle of poverty and lifestyle disruption, together with a high risk of second hits (ie, risk factors such as hypertension, diabetes, obesity, or HIV infection) increase the chance of chronic kidney disease (figure). Optimum maternal and early childhood health and nutrition is mandatory, particularly when considering the link between maternal malnutrition and hypertension, and kidney disease.[14]

Examples such as the Nepal's Safe Motherhood Programme suggest that maternal education, improved use of antenatal care, and maintenance of good health improves health in children.[15] Such studies should be seen as an opportunity rather than a reason to remain inactive.

Moreover, maternal or childhood health promotion has a specific effect on kidney disease. Strategies should focus on primary prevention. Until now governments have not taken the opportunity to intervene in maternal and child promotion in association with future kidney and cardiovascular risk; the link with chronic kidney disease should be emphasised.

As with smoking and obesity, kidney disease will need to be approached as a broad public health issue, associated with chronic illnesses. Similar methods to those used in dealing with smoking and cardiovascular disease are needed.[16–18] Unfortunately there is no radical approach in tackling kidney disease; like cardiovascular disease, kidney disease is not about one risk factor but rather about multiple strategies, which must be sustained. Proposed solutions to manage chronic illnesses should integrate all components of health care effectively.[19] When empowered, women and mothers can substantially enhance the health of their families and communities.[20] This strategy is in keeping with the Millennium Development Goals' call for the efforts to reduce poverty, improve health, particularly for girls and women.[21] When specifically focusing on chronic kidney disease, the words "chronic kidney disease" need to be embedded into future WHO charters and goals for health promotion.

Screening and prevention

In view of the adverse outcomes associated with kidney failure, early detection with screening for chronic kidney disease could prevent progressive loss of renal function and its consequences.[22] This approach can also help with

appropriate dosing of medications, and with prevention of inappropriate exposure to nephrotoxic drugs, and allow timely preparation for dialysis or transplantation. Although screening programmes may be attractive at first glance, only a few lead to net benefits for health, and of these, only a few are economically appropriate. Wilson and Jungner[23] have suggested ten criteria to assess the potential merits of screening programmes. These criteria do not themselves justify the adoption of a screening service, rather, they identify promising areas for further study.

Screening the general population for chronic kidney disease by measuring serum creatinine or albuminuria does not meet several of the Wilson and Jungner criteria (table). Although some attribute reduced incidence of treated kidney failure to a population-based screening programme for chronic kidney disease in Japan,[24] the declining incidence cannot confidently be attributed solely to screening. A recent systematic review reported evidence that screening for chronic kidney disease did not improve clinically relevant outcomes, such as kidney failure, cardiovascular events, and death.[25] Because kidney failure is immediately fatal in much of the developing world, it is easy to speculate that screening should be prioritised in these countries.[26] However, adequate treatment of known non-communicable diseases (previously diagnosed chronic kidney disease, diabetes, hypertension or vascular disease) is more efficient for preventing death and disability than detecting new cases of kidney disease, and so the former should be the priority when resources are limited. Therefore, it would be premature to recommend general population screening for chronic kidney disease in both high-income and low-income countries. Conversely, identifying people at increased risk of chronic kidney disease (so-called selective screening or case finding) meets many of the Wilson and Jungner criteria. This suggests that further study is necessary,[27] particularly in high-risk minority groups.[28] Patients with chronic

	Developed countries		Developing countries	
	General population	Selective screening	General population	Selective screening
1 Condition sought to be an important health problem; the primary aim of screening for chronic kidney disease is to prevent kidney failure and complications of advanced kidney disease such as accelerated atherosclerosis	Yes	Yes	Yes	Yes
2 There should be effective treatment for patients with recognised disease; an important caveat is that several key treatments for chronic kidney disease are also indicated for treatment of non-communicable diseases that cause or frequently coexist with chronic kidney disease. Therefore, detection of chronic kidney disease through screening will not necessarily change management if the other non-communicable diseases have been previously identified	Yes	Yes	Yes	Yes
3 Facilities for diagnosis and treatment should be available	Yes	Yes	No	Not universal
4 There should be a recognisable latent or early stage of the disease; precisely which early stage of chronic kidney disease should be targeted for detection is uncertain. Possibilities include stage 4 chronic kidney disease or stage 3 chronic kidney disease, or heavy albuminuria. The more inclusive the definition, the less likely that criterion number 3 will be met, especially in developing countries or with general population screening	Yes	Yes	Yes	Yes
5 There should be a suitable test; screening for chronic kidney disease should be based on blood tests (estimating equations for glomerular filtration rate in conjunction with serum creatinine assays), urine tests (albuminurias as assessed by dipstick urinalyis or specific assays), or combinations thereof (sequential or simultaneous testing), with or without follow-up testing for confirmation. The optimum testing strategy is unknown and will depend on setting, available resources, and the screened population	Yes	Yes	Yes	Yes
6 The test should be acceptable to the population	Yes	Yes	Yes	Yes
7 The natural history of the disease should be understood; much has been learned about the natural history of chronic kidney disease, but important gaps remain—especially for milder forms of disease, and in people with competing comorbidities. Whether available data about the natural history of chronic kidney disease (largely gathered in developed countries) can be applied to case finding in developing countries (where competing risks, available treatments for comorbidities and the absolute risk associated with chronic kidney disease may vary) is uncertain, especially for the general population. Importantly, how the natural history should influence the ideal frequency for population-based screening (in developed or developing countries) is unknown	No	Yes	No	No
8 There should be an agreed policy on whom to treat as patients; there is general agreement that advanced forms of chronic kidney disease or those with severe albuminuria should be treated, if identified. More remains to be learned about the potential benefits of treating people with very early forms of chronic kidney disease (such as those with normal glomerular filtration rate and persistent mild albuminuria). In view of the large number of such people, the feasibility of screening for and treating these abnormalities is uncertain even if such treatment were known to be helpful	Yes	Yes	Yes	Yes
9 The cost of case finding (including diagnosis and treatment of patients diagnosed) should be economically balanced in association with possible expenditure on medical care as a whole; studies show that screening the general population is not cost effective. Although studies suggest that case finding in developed countries may be economically attractive, these studies have made assumptions that may overestimate benefits. Even in developing countries where the prevalence of chronic kidney disease and other non-communicable diseases is higher than expected (thereby increasing the yield of case finding), it is uncertain whether this represents a wise use of resources, given that treatment of patients with clinically obvious non-communicable diseases (usually more economically efficient) is relatively rare in such settings	No	Yes	No	Uncertain
10 Case finding should be a continuing process and not a once and for all project; the logistical difficulties associated with population-based screening and the large number of cases identified mean that the feasibility of such programmes are uncertain even in developed countries. For the reasons given in criterion 9, the feasibility of ongoing case finding in developed countries is uncertain	Feasibility uncertain	Feasible	Not feasible	Feasibility uncertain

Table: **Wilson-Jungner[23] principles of early disease detection and screening for chronic kidney disease**