A novel class of advanced glycation inhibitors ameliorates renal and cardiovascular damage in experimental rat models

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Abstract

Background. The reno- and cardiovascular-protective effects of angiotensin II receptor blockers (ARBs), have been ascribed, at least in part, to their ability to inhibit the formation of advanced glycation end products (AGEs), independently of their effect on blood pressure. They act through decreased oxidative stress, unlike previously reported AGE inhibitors which entrap reactive carbonyl (RCOs) precursors of AGEs. The hypotensive effects of ARBs', however, may limit their use. In the present study, we report the synthesis of a new AGE inhibitor, TM2002, and its effects in vitro and in vivo.

Methods. We screened a large chemical library (~1300 compounds) including edaravone, a drug used to treat cerebral infarction, for in vitro AGE inhibitory activity. Based upon the structure-function analysis of edaravone derivatives, we synthesized a novel AGE inhibitor, 1-(5-hydroxy-3-methyl-1-phenyl-1H-pyrazol-4-yl)-6-methyl-1,3-dihydro-furo[3,4-c]pyridine-7-ol (TM2002). We delineate in vitro the biological characteristics of TM2002, evaluate in vivo its toxico-pharmacokinetics and document in animal models of rat, their renal and cardiovascular protective effectiveness.

Results. Screening of a large chemical library disclosed that edaravone inhibits in vitro AGE formation efficiently. Unfortunately, like most AGE inhibitors, it also traps pyridoxal, limiting its clinical usefulness. We therefore synthesized a novel AGE inhibitor, TM2002, that does not trap pyridoxal. In vitro, TM2002 shows powerful AGE inhibitory activity.

Markers of oxidation, i.e. o-tyrosine formation and transition metal chelation, are efficiently inhibited by TM2002-like ARBs. TM2002 does not bind to the angiotensin II type 1 receptor. It is readily bioavailable and non-toxic. In vivo, TM2002, given acutely or for 8 weeks, has no adverse effects. In four different rat models of renal injury (anti-Thy1 and ischaemia-reperfusion) and cardiovascular injury (carotid artery balloon injury and angiotensin II-induced cardiac fibrosis), TM2002 improves renal and cardiovascular lesions without modification of blood pressure.

Conclusions. TM2002 is a novel, non-toxic AGE inhibitor acting through ARB-like mechanisms, able to prevent renal and cardiovascular diseases independently of blood pressure lowering.

Keywords: advanced glycation end products; blood pressure; oxidative stress; radical scavenge; renoprotection

Introduction

Antihypertensive inhibitors of the renin-angiotensin system (RAS), such as angiotensin II receptor blockers angiotensin II type 1 receptor blockers (ARBs) and angiotensin-converting enzyme inhibitors (ACEIs), protect the kidney and the heart, at least in part, independently of blood pressure lowering [1–5]. Their clinical use is occasionally limited by an inappropriate hypotension, especially in normo- or hypotensive patients.

We [6–8] and others [9] have recently demonstrated that both in vitro and in vivo ARBs and ACEIs effectively inhibit the formation of advanced glycation end products (AGEs) and suggested that this inhibition mediates tissue protection. The mechanisms involved...
in AGE inhibition differ from those of previously reported AGE inhibitors [6,8]. Aminoguanidine [10], as well as a number of second-generation AGE inhibitors (e.g. OPB-9195 [11], pyridoxamine [12] and LR-90 [13]), trap in vitro reactive carbonyls (RCOs) precursors of AGEs whereas, in contrast, ARBs inhibit RCOs production through decreased oxidative stress and a transition metal chelation [6].

We further hypothesized that inhibitors of advanced glycation and local oxidative stress, devoid of influence on blood pressure, might protect against renal and cardiovascular damages. In the present study, we report the synthesis of such a compound, 1-(5-Hydroxy-3-methyl-1-phenyl-1H-pyrazol-4-yl)-6-methyl-1,3-dihydro-furo[3,4-c]pyridine-7-ol (TM2002), delineate in vitro its biological characteristics, evaluate in vivo its toxicity and pharmacokinetics and, finally, demonstrate in several animal models its renal and cardiovascular protective effectiveness.

Subjects and methods

Reagents

Our chemical library includes 1332 synthetic compounds purchased from Maybridge (Cornwall, United Kingdom), ASINEX (Moscow, Russia), LaboTest (Niederschona, Germany), Specs (Kluiverweg, Netherlands), ENAMINE Ltd. (Kiev, Ukraine), ChemStar (Moscow, Russia), ASDI (Newark, NJ), Sigma (St Louis, MO), Pharmeks (Moscow, Russia), Chemical Diversity Labs (San Diego, CA) and Wako (Osaka, Japan). Olmesartan (Pharmacology and Molecular Biology Research Laboratories, Sankyo Pharmaceutical, Tokyo, Japan), edaravone (Wako), aminoguanidine hydrochloride (Tokyo Chemical Industry, Tokyo, Japan), pyridoxamine (Sigma) and losartan potassium (Wako) were kindly provided or purchased. These compounds were dissolved in DMSO to obtain a stock solution of 50 mM to be further diluted to obtain the required concentration (the final concentrations of DMSO were <10%). We have confirmed that DMSO, at the final concentration of 10%, does not influence the used assays.

Synthesis of TM2002

First, a condensation reaction between pyridoxal and edaravone was carried out. In brief, a solution of pyridoxal hydrochloride (72 mol) in distilled water (200 ml) was added to a solution of edaravone (60 mmol) in 0.1 M of NaOH solution (600 ml). After 1 h reaction at room temperature at pH 9.5, the mixture was acidified with 6 N HCl to pH 5.5 and allowed to precipitate at 4°C overnight. The precipitate was collected and dried overnight in vacuo to give crude TM2002 (60 mmol). The latter was dissolved in 1.6 l of and cooled overnight at 4°C. The filtrate was concentrated to 300 ml under reduced pressure and allowed to precipitate at 4°C. The mixture was acidified with 6 N HCl to pH 5.5. After 1 h reaction at room temperature, 200 ml of 0.1 mM CuSO4 as a catalyst. After the metal-catalysed oxidation reactions were quenched by addition of 1 mM DTPA and 260 units of catalase, excitation-emission wavelength of 275/305 nm.

Inhibition of pentosidine and N\textsuperscript{\textdegree}-carboxymethyllysine generation assays

Fresh heparinized plasma samples were obtained after informed consent from haemodialysis patients prior to the dialysis session. Pooled plasma (n = 11) was incubated with the tested reagents (final concentration of 0.8, 2.0 and 5.0 mM) for 1 week under air at 37°C. The content of pentosidine [14] was analysed on a C18 reverse phase high-performance liquid chromatography (HPLC) as previously described [15]. Synthetic pentosidine was used as a standard.

For quantitation of CML [16], samples (100 µl) were diluted with 100 µl 0.2 M sodium borate (pH 9.1), followed by the reduction for 4 h at room temperature with 20 µl of 1 M NaBH\textsubscript{4} in 0.1 N NaOH. Protein was then precipitated by addition of an equal volume of 20% trichloroacetic acid and pelleted by centrifugation at 2000 x g for 5 min. After the addition of heavy labeled internal standards (d\textsubscript{4}-CML), the sample was hydrolysed in 0.3 ml 6 N HCl at 110°C for 16 h. CML content was measured as its N, O-trifluoroacetyl methyl esters by selected-ion monitoring gas chromatography/mass spectrometry (GC/MS) as described previously [17].

Inhibition of hydroxyl radical-mediated phenylalanine modification

Phenylalanine (1 mM) and the tested compound (final concentration of 0.1, 0.5, 2.5 mM) were dissolved in 200 mM phosphate buffer (pH 7.4) and incubated at room temperature for 4 h with H\textsubscript{2}O\textsubscript{2} (5 mM) in the presence of 0.1 mM CuSO\textsubscript{4} as a catalyst. After the metal-catalysed oxidation reactions were quenched by addition of 1 mM DTPA and 260 units of catalase, α-tirosine was measured by reverse-phase HPLC with a fluorescence detector at excitation-emission wavelength of 275/305 nm.

Transition metal chelating assay

The chelating activity of the tested compounds for transition metal ions was measured by the method of Price et al. [18] with some modifications. Briefly, 15 µl of 50 µM CuCl\textsubscript{2} and
30 μl of tested compound solution were pre-incubated in 1.38 ml phosphate buffer at 30 °C for 5 min. The reaction was initiated by the addition of 75 μl of 10 mM ascorbic acid. Incubation lasted from 0 to 60 min at 30 °C so as to allow kinetic reaction studies. Ascorbic acid content was determined by reverse phase HPLC with absorbance detection at 244 nm.

**Entrapment of RCOs and pyridoxal formation**

Three ζ-dicarbonyl compounds, i.e. glyoxal (Sigma), methylglyoxal (Sigma) and 3-deoxyglucosone (Dojindo Laboratories, Kumamoto, Japan), were used as RCOs. These compounds (each final concentration of 0.1 mM) and the tested compound (final concentration of 0.5, 1.0, 3.0 mM) were dissolved in 100 mM phosphate buffer (pH 7.4) and incubated at 37 °C for 30 min. Then, the incubation mixture (70 μl) reacted for 2 h at room temperature with 2 μl of 1 mM 2,3-butanedione (internal standard), 68 μl of distilled water, 56 μl of 2 M perchloric acid and 28 μl of 1% o-phenylenediamine. The final content of quinoxalines (ζ-dicarboxyls phenylenediamine derivatives) and pyridoxal formed during incubation was measured by C18 reverse-phase HPLC with absorbance detection at 315 nm and at 292 nm, respectively [19].

**Pyridoxal 5'-phosphate entrapment**

The tested compounds (0.5 mM) were incubated, for 0–10 h, with pyridoxal 5'-phosphate (50 μM) (Wako) in phosphate buffered saline at 25 °C. Aliquots were removed at various times and pyridoxal 5'-phosphate content was assayed by reverse-phase HPLC with a fluorescence detector at excitation-emission wavelength of 300/400 nm [6]. The final content of pyridoxal, detected as a distinct peak from pyridoxal 5'-phosphate, was also determined.

**Angiotensin II receptor binding assay**

The membrane fraction of Chinese hamster ovary (CHO) cells stably transfected with the human angiotensin type 1 receptor was purchased (Biosignal A: Packard Bioscience Company, Billerica, MA). Binding experiments were performed by incubation, for 2 h at room temperature, the membrane fraction (5 μg/well), [125I]angiotensin II (0.1 nmol/well, specific activity: 2000 Ci/mmol) (Amersham Biosciences, Uppsala, Sweden) and a tested compound in a buffer solution containing 20 mM Tris–HCl (pH 7.4), 120 mM NaCl, 5 mM MgCl2, 0.05% bovine serum albumin, 1 μM (p-aminophenyl) methanesulfonyl fluoride hydrochloride (Wako), 0.5 mM EDTA and 0.1 mM dithiothreitol. After incubation, the mixture was immediately filtered through a GF/C filter presoaked with 0.3% polyethyleneimine to separate membrane-bound from free radioligands. The radioactivity on the filter was counted by a gamma counter. Specific binding was determined as the difference between binding in the absence and presence of 1 μM unlabelled angiotensin II.

**Cellular toxicity assay**

HeLa cells were cultured for 24 h in the Dulbecco’s MEM in the presence of the tested compound (10–100 μM). Cytotoxicity was determined by the release of lactate dehydrogenase (LDH) in the culture medium measured with a kit (Promega, Madison, WI) and expressed as percentage of the total LDH activity released after freeze and thaw lysis of all culture cells.

**Acute and chronic toxicities**

TM2002 (500, 1000, 1500, 2000 and 2500 mg/kg) was given by oral gavage to Institute of Cancer Research (ICR) mice weighing 30–35 g (CLEA JAPAN, Tokyo, Japan). Two weeks later, each mouse was weighed and evaluated histologically and biochemically.

TM2002 (100 mg/kg, twice a day) was also administrated by oral gavage to male Wistar rats (CLEA JAPAN) weighing 180–200 g for 8 weeks. At the end of the study, they were weighed and evaluated.

**Pharmacokinetics study in rats**

TM2002 (50 mg/kg) was administrated by oral gavage to male Wistar rats weighing 180–200 g. Heparinized blood samples were collected from the vein before (0 h) and at 1, 2, 6 and 24 h after administration. TM2002 concentration in the plasma was determined by reverse-phase HPLC with absorbance detection at 270 nm. Maximum drug concentration time (Tmax), maximum drug concentration (Cmax) and drug half-life (T1/2) were calculated.

**Subacute toxicity and vitamin B6 kinetics in rats**

TM2002 (200 mg/kg/day) was administrated by oral gavage to male Wistar rats weighing 180–200 g for 2 weeks. Heparinized blood samples were collected from the vein before (0 h) and at 6 h, 6 days and 15 days after administration. Three forms of vitamin B6 (pyridoxin, pyridoxal and pyridoxamine) were measured in plasma samples by reverse phase HPLC. Blood samples were used for other biochemical assays (glucose, total cholesterol, triglyceride, GGT, GPT, creatinine, urea nitrogen, total protein and albumin).

**Renal injury models**

The renoprotective effect of TM2002 was evaluated in two renal injury rat models, anti-Thy1 nephritis and ischemia-reperfusion renal injury. The protocol was in accordance with the Animal Experimentation Guidelines of University of Tokyo.

Anti-Thy1 nephritis was produced in male Wistar rats weighing 150–170 g. After the intravenous injection of an IgG (OX-7) mouse monoclonal anti-Thy1.1 antibody (1 mg/kg body weight) on day 0, the animals were randomly divided into four groups and given by oral gavage, from day 1 to 5, carboxymethylcellulose (CMC) containing the following agents: vehicle (n = 5), TM2002 (50 mg/kg BW twice a day, n = 6), edaravone (27 mg/kg BW twice a day, n = 6) and both edaravone (27 mg/kg BW twice a day) and pyridoxal (30 mg/kg BW twice a day, n = 6). On day 6, the animals were
euthanized, blood was drawn by cardiac puncture and the kidneys were removed for histological analysis.

Ischaemia-reperfusion renal injury was produced in male Wistar rats weighing 150–170 g. On day 1, a right nephrectomy was performed after ligation of the renal pedicle and the ureter. On day 0 ischaemic injury was induced by clamping the left renal artery and vein for 45 min. Core body temperature was maintained at 37°C using a homeothermic table during surgery. The animals were randomly divided into four groups given the same agents as described above (vehicle, n = 3; TM2002 50 mg/kg, twice a day, n = 7; edaravone 27 mg/kg, twice a day, n = 7; combination of edaravone 27 mg/kg and pyridoxal 30 mg/kg, twice a day, n = 6). The agents were given daily from day 0, after the anesthesia until death on day 2. 48 h after injury. Blood was drawn by cardiac puncture and the kidneys were removed for histological analysis.

For morphological analysis, coronal sections of renal tissue (4 μm thick) were stained with periodic acid-Schiff (PAS) and examined by light microscopy in a blinded fashion. In the anti-Thy1 nephritis model, glomerular hypercellularity was evaluated in 50 glomeruli, selected randomly in each animal. The number of cells was counted in each glomerulus and averaged. In the ischaemia-reperfusion model, tubulointerstitial damage was scored in a blinded manner in more than 15 randomly selected fields of cortex per cross section. Injury was graded (0–4) on the basis of the percentage of tubular cellularity, basement fields of cortex per cross section. Injury was graded (0–4) in a blinded manner in more than 15 randomly selected fields of cortex per cross section. Injury was graded (0–4+)

Cardiovascular injury models

The cardiovascular protective effect of TM2002 was evaluated in two rat models, carotid artery balloon injury and angiotensin II-induced cardiac fibrosis model. The protocol was in accordance with the Animal Experimentation Guidelines of Tokai University School of Medicine.

For carotid artery balloon injury, male 10-week-old Sprague-Dawley rats were used. Endothelial denudation and injury to the vascular wall were produced in the left common carotid artery of rats as described previously [20]. Rats were given orally by gavage, twice daily, for 14 days after injury, either vehicle (n = 10) or 50 mg/kg of TM2002 suspended in 0.5% CMC sodium salt solution (n = 10). Both carotid arteries were excised 14 days after the balloon injury and adventitia removed with a forceps and washed three times in PBS. Sections (5 mm length) of the carotid artery were fixed and sectioned for light microscopy for morphometric analysis. The areas between the external elastic lamina and the internal elastic fiber as well as the vessel lumen were measured by micrometry on microscopic photographs of each vessel. Areas of neointima and media and the ratios of neointima to either media or to whole vessel were calculated.

Angiotensin II-induced cardiac fibrosis was produced in 20 male 6-week-old Wistar rats by the infusion of angiotensin II (Wako) for 2 weeks at the rate of 0.626 mg/kg/day, by an osmotic mini pump (Alza Pharmaceutical, Palo Alto, CA). Rats were randomly divided into four groups (n = 5 each) and given dose an oral dose of either vehicle, 25 mg/kg losartan, 100 mg/kg TM2002, or 200 mg/kg TM2002 twice a day. A control group (n = 5) received only vehicle. Systolic BP was determined in conscious rats by the tail-cuff method at the end of study. Significant differences >10 mmHg can only be obtained for tail-cuff BP determinations, given inter- and intrindividual variability. The severity of myocardial fibrosis was quantified according to the method of Numaguchi et al. [21]. The fibrosis areas were identified in whole coronal cardiac sections stained with Masson’s trichrome (MT) and were calculated by an ImageJ software (NIH). The score of fibrosis was expressed as the ratio to myocardial fibrosis in age- and sex-matched control rats.

Statistical analysis

Differences among groups were assessed by Kruskal–Wallis test. The statistical significance was determined by Mann–Whitney U-test. Comparisons of myocardial fibrosis or SBP were performed by one-way ANOVA followed by Bonferroni multiple-comparisons procedures for comparisons of pairs of means. A P-value of <0.05 was considered to indicate statistical significance; all tests were two-tailed. All statistical analyses were performed on the statistical package SPSS for Windows (Version 14.0, SPSS, Chicago, USA).

Results

Identification of edaravone as a potent AGE inhibitor

We screened a chemical library, containing 1332 synthetic compounds, for AGE inhibition by an in vitro assay performed in pooled uremic plasma. Among the tested compounds, edaravone (Figure 1A), a drug used for the treatment of cerebral infarction [22], displayed distinctive characteristics.

It inhibited in vitro the production of two AGEs, pentosidine and CML (Table 1). Inhibition strikingly exceeded that achieved by previously reported AGE inhibitors, e.g. aminoguanidine, pyridoxamine and olmesartan (ARB). Even at much lower drug concentrations, ranging from 0.01 to 800 μM, the dose response was curvilinear (data not shown).

Edaravone also reduced oxidative stress. Unlike aminoguanidine, it inhibited o-tyrosine formation during hydroxyl radical-mediated phenylalanine modification, a specific and sensitive assay for hydroxyl radical-induced protein damage (Figure 2). Inhibition was within the range reported for ARBs.

Edaravone chelated transition metal ions involved in the Fenton reaction that generates hydroxyl radicals. Again, unlike aminoguanidine, it inhibits copper-catalysed oxidation of ascorbic acid. Inhibition is slightly less effective than that observed for ARBs (Table 2).

Finally, unlike ARBs but like aminoguanidine and OPB-9195, edaravone trapped both reactive carbonyl compound (RCOs) precursors for AGEs (Figure 3A) and unfortunately, pyridoxal 5’-phosphate (Figure 3B). This latter effect indeed precludes its long-term use in man. These results are surprising as edaravone
lacks the RCO-entrapping structures shared by most, if not all, previously tested AGE inhibitors, i.e. amino, guanidino or hydrazine groups.

\[ \alpha \]-Methylene group, a potent binder for RCOs and pyridoxal

Study of the structure-function relationship of various edaravone derivatives led to the discovery of the as yet unreported key role played by the \( \alpha \)-methylene group (\( \alpha \)-position carbon of carbonyl on the ring indicated by broken circle in Figure 1A) in pyridoxal entrapment. All derivatives sharing the \( \alpha \)-methylene group trapped RCOs as well as pyridoxal as shown in Figure 1B for some of the 21 tested derivatives (glyoxal trapping is used as a surrogate for RCOs). In contrast, \( \alpha \)-methylene modified derivatives had no such effect (Figure 1C).

Development of a novel synthetic compound, TM2002

The \( \alpha \)-methylene group of edaravone was modified by the aldol condensation reaction. Reaction of edaravone with a variety of carbonyls yielded 18 derivatives some of which are shown in Figure 1D. Their AGE lowering activity proved better than that of edaravone (Figure 1). 1-(5\(^{\prime}\)-hydroxy-3\(^{\prime}\)-methyl-1\(^{\prime}\)-phenyl-1'H-pyrazol-4\(^{\prime}\)-yl)-6-methyl-1, 3-dihydrofuro [3,4-c]
pyridine-7-ol (TM2002) was eventually retained for further investigation as its synthesis relied on aldol condensation of edaravone with pyridoxal, a natural, safe substance (i.e. vitamin B6). The aldol reaction is reversible so that TM2002 might release subsequently equimolar amounts of the original edaravone and pyridoxal, precluding pyridoxal depletion.

Inhibition of advanced glycation and oxidative stress by TM2002

TM2002 inhibited the in vitro production of two AGEs, pentosidine and CML, to the same extent or even better than edaravone (Table 1). It inhibited o-tyrosine formation during hydroxyl radical-mediated phenylalanine modification as efficiently as edaravone (Figure 2). Its chelating activity for transition metal ions was similar to that of edaravone (Table 2). RCOs (Figure 3A) and pyridoxal 5’-phosphate (Figure 3B) entrapment was significantly lower with TM2002 than with edaravone. Of note, the partial transformation of TM2002 during incubation released free edaravone able to entrap mildly RCOs and pyridoxal 5’-phosphate while pyridoxal level rose only after incubation with TM2002 but not with the other tested compounds (Figure 3B).

TM2002, just as ARBs and edaravone, is a potent inhibitor of advanced glycation and oxidative stress but, contrary to edaravone, it is unlikely to produce pyridoxal-related side effects.

No affinity of TM2002 with an angiotensin II type 1 receptor

In the competition assay for binding to human angiotensin II type 1 receptor, TM2002 had no specific binding affinity up to a concentration of 10 μM (IC50 of olmesartan and angiotensin II in this assay was 1.91 and 0.56 nM, respectively).

Toxicity and pharmacokinetics of TM2002

Cellular injury was assessed in vitro in HeLa cells as the LDH activity released into the culture medium after 24 h. TM2002 had a very low toxicity: at the concentration of 100 μM, the maximum rise in LDH activity with TM2002 (18.2 ± 0.8%) was equal to that of controls (21.8 ± 2.5%).

Acute in vivo toxicity of TM2002 was evaluated in mice after a single dose ranging from 500–2500 mg/kg. Blood pressure was unchanged and no symptoms were observed up to 2 weeks after administration. Rats given daily 200 mg/kg of TM2002 for 8 weeks exhibited no abnormal serum and urine biochemistries. TM2002 did not affect the haemoglobin levels: 14.3 ± 0.5 g/dl in vehicle-treated rats vs 13.8 ± 0.6 g/dl in TM2002-treated rats (no statistical significance). Rats given TM2002 did not develop anaemia.

Pharmacokinetics of TM2002, assessed in rats given an oral dose of 50 mg, revealed calculated plasma Tmax, Cmax and T1/2 of 1 h, 1.9 μg/ml, and 0.5 h, respectively.

Rats given 200 mg/kg/day of TM2002 for 2 weeks had no adverse effects (final plasma concentration: 0.1 ± 0.0 μg/ml, n = 5). Unlike animals given edaravone or vehicle, their plasma pyridoxal (Figure 4A) and

### Table 1. Half-maximal inhibition (IC50) value of pentosidine or N’-carboxymethyllysine (CML) formation during incubation of uraemic plasma

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 (mM)</th>
<th>Pentosidine</th>
<th>CML</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminoguanidine</td>
<td>9.84</td>
<td>1.76</td>
<td></td>
</tr>
<tr>
<td>Olmesartan</td>
<td>2.81</td>
<td>5.42</td>
<td></td>
</tr>
<tr>
<td>Pyridoxamine</td>
<td>5.27</td>
<td>5.09</td>
<td></td>
</tr>
<tr>
<td>Edaravone</td>
<td>0.76</td>
<td>1.44</td>
<td></td>
</tr>
<tr>
<td>TM2002</td>
<td>&lt;0.5</td>
<td>1.41</td>
<td></td>
</tr>
</tbody>
</table>

Pooled plasma from haemodialysis patients was incubated with the tested compounds (final concentration of 0.8, 2.0 and 5.0 mM) for 1 week under air at 37 °C. The yields of pentosidine and CML in the incubation mixtures were determined by HPLC and gas chromatography/mass spectrometry (GC/MS), respectively.

### Table 2. Half-maximal inhibition (IC50) value of copper-catalysed oxidation of ascorbic acid by the tested compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminoguanidine</td>
<td>&gt;4000</td>
</tr>
<tr>
<td>Olmesartan</td>
<td>4.5</td>
</tr>
<tr>
<td>Pyridoxamine</td>
<td>1749</td>
</tr>
<tr>
<td>Edaravone</td>
<td>114</td>
</tr>
<tr>
<td>TM2002</td>
<td>84</td>
</tr>
</tbody>
</table>

Ascorbic acid (500 μM) was incubated at 30 °C with 500 nM CuCl₂ in 50 mM phosphate buffer (pH 7.4) in the presence of several concentrations of the tested compound. The contents of ascorbic acid in the incubation mixtures were determined by HPLC.

Fig. 2. Reduction of oxidative stress. Inhibition of o-tyrosine formation during hydroxyl radical-mediated phenylalanine modification. After the metal-catalysed oxidation reactions, o-tyrosine was measured by reverse-phase HPLC. Data are expressed as means ± SD. * P < 0.05 vs control by Mann Whitney U-test.

No affinity of TM2002 with an angiotensin II type 1 receptor

In the competition assay for binding to human angiotensin II type 1 receptor, TM2002 had no specific binding affinity up to a concentration of 10 μM (IC50 of olmesartan and angiotensin II in this assay was 1.91 and 0.56 nM, respectively).

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Pharmacokinetics of TM2002, assessed in rats given an oral dose of 50 mg, revealed calculated plasma Tmax, Cmax and T1/2 of 1 h, 1.9 μg/ml, and 0.5 h, respectively.

Rats given 200 mg/kg/day of TM2002 for 2 weeks had no adverse effects (final plasma concentration: 0.1 ± 0.0 μg/ml, n = 5). Unlike animals given edaravone or vehicle, their plasma pyridoxal (Figure 4A) and
pyridoxamine (Figure 4B) levels rose with time. The former was attributed to the dissociation of TM2002 into edaravone and pyridoxal while the latter was taken to reflect the in vivo enzymatic conversion of the released free pyridoxal. Plasma pyridoxin levels remained below the detection limit (<3.0 ng/ml) in all tested animals, as anticipated from the fact that pyridoxin is not formed from pyridoxal in vivo. Serum biochemistry in rats given TM2002 revealed no abnormality.

TM2002 emerges thus as a safe compound whose metabolites, edaravone, pyridoxal and pyridoxamine, have been proved non-toxic (a medical agent or natural substances).

Renoprotection of TM2002
In the anti-Thy1 mesangioproliferative nephritis model, vehicle-treated animals developed severe

Fig. 3. Entrapment of RCOs, pyridoxal, and pyridoxal 5'-phosphate. (A) The 100 mM phosphate buffer (pH 7.4) containing glyoxal, methylglyoxal and 3-deoxyglucosone (each final concentration of 0.1 mM) was incubated for 30 min at 37°C with the tested compound (final concentration of 0.5, 1.0, 3.0 mM), followed by the determination of the final content of RCOs (quinoxaline derivatives) and pyridoxal by reversed phase HPLC. Data are expressed as means ± SD. *P < 0.05 vs control by Mann Whitney U-test. (B) Kinetics of pyridoxal 5'-phosphate entrapment and pyridoxal formation by the tested compounds during incubation. Pyridoxal 5'-phosphate (50 μM) was incubated with the tested compounds (500 μM) in PBS at 37°C. Aliquots were removed at various times and assayed for residual pyridoxal 5'-phosphate and pyridoxal on a reverse-phase HPLC. The pyridoxal was formed during incubation with compounds other than TM2002.
Renoprotection provided by TM2002 was also assessed in the ischaemia-reperfusion acute kidney failure model (Table 4 and Figure 5B). Vehicle-treated animals developed severe kidney failure with elevated BUN (135 mg/dl) and severe tubulointerstitial injury (score of three vs nil in controls). Edaravone alone or in combination with pyridoxal did not significantly modify BUN but increased the kidney injury score less (P < 0.05). An equimolar amount of TM2002 (100 mg/kg/day) provided significant renoprotection: the increase in BUN and the kidney score in response to TM2002 was 24 and 12%, respectively, of that in response to vehicle (P < 0.05). These effects were significantly superior to edaravone (P < 0.05) and to edaravone plus pyridoxal (P < 0.01). Here again, blood pressure was unchanged by TM2002 treatment.

Cardiovascular protection of TM2002

Effectiveness of TM2002 was investigated in a carotid artery balloon-injury rat model (Table 5 and Figure 6A). In rats given vehicle, the left carotid artery trauma resulted after 14 days in a remarkable intimal thickening with an attendant rise in the intima/media ratio (P < 0.001) in comparison with the contralateral non-injured artery. TM2002 (100 mg/kg/day for 14 days) decreased intimal thickening and reduced by more than 28% (P < 0.05) of the intima/media ratio of the injured carotid artery. Blood pressure was not modified (122 ± 3 mmHg in control rats vs 120 ± 2 mmHg in TM2002 rats). The non-injured contralateral right carotid artery was identical in TM2002-treated and control rats.

Cardiovascular protection of TM2002 was also evaluated in a model of angiotensin II-induced cardiac fibrosis (Table 6 and Figure 6B). Angiotensin II infused during 2 weeks, raised blood pressure in comparison with control rats given vehicle (P < 0.001). The systolic blood pressure was increased by 106 mmHg in response to angiotensin II infusion. Losartan (50 mg/kg/day) given simultaneously maintained blood pressure in the normal range whereas neither low nor high dose TM2002 modified the elevated blood pressure. Angiotensin II infusion in rats severely raised the cardiac fibrosis score (P < 0.05) almost 4-fold above that of the control group given vehicle only. The limited amelioration of the fibrosis score induced by losartan failed to reach statistical significance. In contrast, despite its lack of effect on blood pressure, TM2002 (200 and 400 mg/kg/day) reduced the cardiac fibrosis score by 33 and 64%, respectively, only the latter change reaching statistical significance (P < 0.05). The fibrosis score was equivalent in the high dose TM2002 and the control rats given only vehicle.

Discussion

The present procedure leading to the synthesis of TM2002 and the identification of a new class of AGE inhibitors is original. First, a large library of more than a thousand chemical compounds including currently used medical drugs, was screened by high-throughput for an AGE-lowering profile. Edaravone, a drug used in man to treat cerebral infarction, emerged as more powerful than all previously known AGE inhibitors. In a second step, its characteristics were assessed in vitro. Unfortunately, it was discovered that it traps pyridoxal, thus limiting thus its long-term use in man.
Still, edaravone did not contain any of the pyridoxal trapping sites previously identified in other AGE-lowering drugs. Structure-function analysis of edaravone derivatives led to the discovery that an \( \alpha \)-methylene group was responsible for pyridoxal (and RCO) binding. In a last step, the \( \alpha \)-methylene group was modified by aldol condensation with several compounds. TM2002 was eventually identified and

**Table 3. Renoprotection of TM2002 in the anti-Thy1 mesangioproliferative nephritis model**

<table>
<thead>
<tr>
<th>Mesangioproliferative glomerulonephritis (cells/glomeruli)</th>
<th>Anti-Thy1 nephritis rats</th>
<th>Control rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>106.5 ± 5.5(^{f})</td>
<td>62.8 ± 2.7(^{e,c})</td>
</tr>
<tr>
<td>Edaravone</td>
<td>82.3 ± 1.4(^{e})</td>
<td>60.3 ± 2.8</td>
</tr>
<tr>
<td>Edaravone+pyridoxal</td>
<td>83.5 ± 3.1(^{b})</td>
<td></td>
</tr>
<tr>
<td>TM2002</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SE.

\(^{a}\)P < 0.001 by Kruskal–Wallis test (vehicle, edaravone, edaravone+pyridoxal, TM2002).

\(^{b}\)P < 0.05, \(^{c}\)P < 0.01 vs vehicle by Mann–Whitney U-test.

\(^{d}\)P < 0.01 vs edaravone by Mann–Whitney U-test.

\(^{e}\)P < 0.01 vs edaravone+pyridoxal by Mann–Whitney U-test.

\(^{f}\)P < 0.01 vs control (normal rats) by Mann–Whitney U-test.

**Table 4. Renoprotection of TM2002 in the ischaemia-reperfusion acute kidney failure model**

<table>
<thead>
<tr>
<th>Ischaemia-reperfusion acute kidney failure rats</th>
<th>Control rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>135.0 ± 20.7(^{f})</td>
</tr>
<tr>
<td>Edaravone</td>
<td>151.0 ± 32.9</td>
</tr>
<tr>
<td>Edaravone+pyridoxal</td>
<td>86.3 ± 25.7</td>
</tr>
<tr>
<td>TM2002</td>
<td>46.2 ± 8.6(^{e,d})</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SE.

\(^{a}\)P < 0.05, \(^{b}\)P < 0.001 by Kruskal–Wallis test (vehicle, edaravone, edaravone+pyridoxal, TM2002).

\(^{c}\)P < 0.05 vs vehicle by Mann–Whitney U-test.

\(^{d}\)P < 0.05 vs edaravone by Mann–Whitney U-test.

\(^{e}\)P < 0.01 vs edaravone+pyridoxal by Mann–Whitney U-test.

\(^{f}\)P < 0.05 vs control (normal rats) by Mann–Whitney U-test.

Fig. 5. Renal tissue protection of TM2002 in two experimental rat models. (A) anti-Thy1 nephritis model. Renal tissues from vehicle-treated (upper panel) and TM2002-treated (lower) rats. Magnification, ×400. (B) ischaemia-reperfusion acute renal injury model. Vehicle treated (upper panel) and TM2002-treated (lower rats). Magnification, ×200. Note that TM2002 (100 mg/kg/day) given in rats significantly improved glomerular hypercellularity (A) and tubulointerstitial injury (B).
Table 5. Improvement by TM2002 of neointimal formation in the carotid artery after balloon injury rat model

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Intima (mm²)</th>
<th>Media (mm²)</th>
<th>Intima/media ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Left</td>
<td>Right</td>
<td>Left</td>
<td>Right</td>
</tr>
<tr>
<td>Vehicle</td>
<td>0.13 ± 0.01a</td>
<td>0.00 ± 0.00</td>
<td>0.14 ± 0.01a</td>
</tr>
<tr>
<td>TM2002 (50 mg/kg, twice a day)</td>
<td>0.10 ± 0.01b</td>
<td>0.00 ± 0.00</td>
<td>0.15 ± 0.01b</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SE.

*P < 0.001 vs non-injured right carotid artery (vehicle) by Mann–Whitney U-test.

*P < 0.001 vs non-injured right carotid artery (TM2002) by Mann–Whitney U-test.

*P < 0.05 vs injured left carotid artery (vehicle) by Mann–Whitney U-test.

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Fig. 6. Cardiovascular tissue protection of TM2002 in two experimental rat models. (A) Carotid artery balloon injury rat model. Carotid arterial tissues obtained after balloon injury from injured left (left) and non-injured contralateral right (middle) arteries of vehicle-treated rats, and from injured left artery of TM2002-treated rats (right). Magnification, ×25. (B) angiotensin II induced cardiac fibrosis model. Heart tissues from angiotensin II-infused (upper left) and non-infuse (upper right) rats on vehicle, and from angiotensin II-infused rats on losartan (lower left) and TM2002 (lower right). Magnification, ×100.
Systolic blood pressure (mmHg) 238

Data are expressed as mean ± SE.

Table 6. Protection of TM2002 in the angiotensin II-induced cardiac fibrosis model

<table>
<thead>
<tr>
<th>Angiotensin II-infused rats</th>
<th>Control rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>Losartan 50 mg/kg/day</td>
</tr>
<tr>
<td>Myocardial fibrosis&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.6 ± 0.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>238 ± 6&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

In vitro testing demonstrated that TM2002 acts through mechanisms different from those of other AGE inhibitors. Aminoguanidine [10] and OPB-9195 [11], i.e. hydrazine and/or guanidine derivatives, inhibit AGE formation by trapping their RCO precursors as well as pyridoxal. Pyridoxamine [12] also traps RCO precursors but not pyridoxal. The recently reported LR-90<sup>13</sup> traps RCO precursors but its interaction with pyridoxal remains unknown. In contrast, ARBs (and ACEIs) do not trap RCO precursors but reduce their production through an inhibition of oxidative stress (i.e. hydroxyl radical scavenging and inhibition of the Fenton reaction) [6]. TM2002 acts through ARB-like mechanisms. It does not trap RCO precursors and pyridoxal but reduces oxidative stress. Unlike ARBs, it lacks specific binding affinity to the human angiotensin II type 1 receptor in vitro and therefore does not alter blood pressure in vivo.

TM2002 is orally bioavailable as demonstrated by its pharmacokinetics. It is also toxicologically safe. In vitro, its cytotoxicity is very low. In mice, a single dose of up to 2.5 g/kg at one time, proved nontoxic. In rats, 200 mg/kg/day for 8 weeks, did not result in observable toxicity; after 2 weeks, it not only failed to decrease plasma pyridoxal levels but rather increased them with a simultaneous rise of pyridoxamine. TM2002 is safe and its metabolites, edaravone, pyridoxalpyridoxamine, appear to be non-toxic in the short term. Whether any long-term toxicity exists remains to be seen in future studies.

The relevance of TM2002 for the pursuit of clinical goals remains to be discussed. The first issue relates to the role of blood pressure reduction in the renal protective effects of RAS inhibitors such as ARBs. We hypothesized that ARBs act at least in part, through a reduction of local oxidative stress and advanced glycation [6–8]. The availability of a powerful AGE and oxidative stress inhibitor devoid of influence on angiotensin now allows the evaluation of this hypothesis. TM2002 indeed protects the kidney in two experimental rat models, despite its lack of effect on blood pressure.

The anti-Thy1 mesangiproliferative glomerulonephritis model is characterized by severe glomerular proliferation without hypertension. TM2002 returned glomerular cellularity to control levels, without influencing blood pressure.

The ischaemia reperfusion acute kidney failure model is completely different: it mixes renal failure with severe tubulointerstitial injury. TM2002 improved significantly both renal function and the kidney injury score, here again without changes in blood pressure.

The in vivo effectiveness of TM2002 illustrates the renoprotective benefits likely accruing from AGE and oxidative stress inhibition, independent of blood pressure lowering. This conclusion fits with the renoprotection afforded by AGE inhibitors in diabetic rats without hypertension [9,23] or by ACEIs and ARBs in normotensive human diabetics [24,25]. It also supports Monnier’s [26] contention that AGE accumulation and its pathological consequences could be corrected by agents with potent hydroxyl radical scavenging and transition metal chelating activities. However, further investigation in typical AGE-associated disease models, e.g. diabetic animals, will be necessary to support this view.

We extend the protective role of TM2002 to experimental cardiovascular injury generated in two rat models. We previously demonstrated that
advanced glycation was implicated in the post-traumatic proliferation of the carotid neo intima [20]. TM2002 reduced intimal thickening by more than 28%, without changes in blood pressure. The model of angiotensin II-induced cardiac fibrosis also provided unexpected insights. In this model, hypertension is associated with marked cardiac fibrosis evaluated by a histological score. TM2002 reduced dose-dependently without changes in the prevailing hypertension. In contrast, losartan returned blood pressure to normal without changes in the cardiac fibrosis score.

Finally, it should be mentioned that TM2002 also improved focal cerebral ischaemic injury produced in two different experimental rat models, i.e. transient focal ischaemia induced by thread occlusion and permanent focal ischaemia induced by photothrombotic occlusion (submitted for publication).

Again these data highlight that benefits accruing from the inhibition of advanced glycation and oxidative stress develop independently of blood pressure changes. In this regard, TM2002 compares favourably with its parent molecules. It proved superior to equimolar amounts of edaravone, with or without added pyridoxal, in the prevention of anti-Thy1 glomerulonephritis and of ischaemia-reperfusion interstitial nephritis. Of note, TM2002 was also significantly more efficacious than edaravone in the rat focal ischaemic cerebral model (submitted for publication).

Our results allow an evaluation of the renoprotec- tion achieved by pyridoxamine in diabetic rat models [27,28]. Both in the anti-Thy1 glomerulonephritis and in the ischaemic reperfusion acute renal failure models, neither pyridoxal nor pyridoxamine (our unpublished observation) at the dose of 60 mg/kg BW (corresponding to the equal molar of TM2002 used in this study) exhibited any protective effect. Furthermore, the combination of pyridoxal with edaravone did not augment the latter’s effectiveness (Tables 3 and 4).

In conclusion, by a high-throughput screening of the AGE-lowering profile of more than a thousand chemical compounds and medicinal chemistry, we synthesized a novel, non-toxic, effective AGE inhibitor acting by mechanisms different from those of previously identified AGE inhibitors. Our newly synthesized AGE inhibitor has unique tissue protective properties probably due to its capacity to inhibit advanced glycation together with oxidative stress. It may open new avenues to treat various diseases, in which advanced glycation and oxidative stress are implicated.

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Conflict of interest statement. None declared.

References


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