Tissue concentration and heart/plasma ratio measurement

After receiving single intravenous administration of DPP-4 inhibitor (3 mg/kg), the rats were sacrificed either 1 hour or 4 hours after (n = 3 each); at each time point, we collected approximately 200 μL of blood from the saphenous vein as well as the heart. All blood samples were transferred into micro-centrifuge tubes containing anti-coagulant (4 μL of 0.5 M K_2EDTA) and placed on wet ice; within 30 minutes after sampling, the blood samples were processed for plasma by centrifugation at 3000 g for 15 minutes at 4 ℃. The plasma samples were transferred to polypropylene tubes, quick frozen over dry ice, and kept at −70 ℃ until LC-MS/MS analysis.

LC-MS/MS methods for quantitative determination of test compounds in the corresponding biological matrix were developed under non-GLP compliance. A calibration curve with 8 non-zero calibration standards was applied for the method including LLOQ. A set of QC samples consisting of low, middle, and high concentrations were applied for the method. The study sample analysis was performed concurrently with a set of calibration standards and two sets of QC samples using the LC-MS/MS method. Finally, the heart/plasma ratio (H/P ratio) of each drug at 4 hours after a single intravenous injection was calculated from their respective absolute tissue and plasma concentrations.

In vitro anti-calcifying efficacy test

Human aortic VICs were prepared from the human aortic valve cusps from patients with severe AS by enzyme isolation and maintained as previously described.[1,2] Briefly, human VICs were grown in Dulbecco's Modified Eagle's Medium (DMEM, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific), penicillin (100 U ml-1, Life Technologies), and streptomycin sulfate (Thermo Fisher Scientific), as previously described.[3]

Primary cultured human VICs (passage 3-5) were used to evaluate the anti-calcifying

efficacy of the DPP-4 inhibitors according to a previously described method.[4] To induce osteogenic differentiation of primary human VICs, 0.25 mM L-ascorbic acid, 10 mM β-glycerophosphate, and 10 nM dexamethasone were added to complete medium (DMEM, Thermo Scientific).[5] The osteogenic medium was changed every 3 days. After a week of osteogenic stimulation, anti-calcification efficacy was determined by alkaline phosphatase (ALP) activity according to previously described protocol[5] in VICs which were treated with five different DPP-4 inhibitors. Using these measure, half of the maximal effective concentration (EC50) for anti-calcification was calculated from quintuplicate 12-point dose-response curves for each DPP-4 inhibitors. Measurements were made under optimized conditions to generate consistent and reproducible data that reliably reflects the drug's potency. The inhibitor concentration, expressed in molar units (M), against the percent of control activity is plotted. Using the linear ($y=mx+n$) or parabolic ($y=ax^2+bx+c$) equation on this graph for $y=50$ value x point becomes EC50 value.

Propensity score matching

Propensity scores were estimated without regard to the outcome variables, using multiple logistic-regression analysis. Supplemental Table 1 shows baseline clinical characteristics before and after propensity matching. C-statistic for the logistic regression model was 0.68. The propensity score-matched pairs were created by matching patients who received favourable DPP-4 inhibitors and those without (1:2 matching) using calipers of width equal to 0.2 of the standard deviation of the logit of the propensity score. We employed the standardized mean difference (SMD) to check for differences in baseline characteristics. It has been suggested that a SMD of < 10% probably denotes a negligible imbalance.

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Supplemental Table 1 Pharmacokinetic and pharmacodynamic profiles of five DPP-4 inhibitors used for patients in this study

	Linagliptin (X1)	**Gemigliptin (X2)**	**Alogliptin (Y1)**	**Sitagliptin (Y2)**	**Vildagliptin (Y3)**
Anti-calcifying EC50 (ng/mL)	316.1	627.3	369.9	284.7	8213.0
Heart tissue concentration at 1 hr (ng/g)	827.0±197.1	2133.3±232.9	746.3±50.1	749.0±102.5	84.8±14.2
Heart tissue concentration at 4 hr (ng/g)	139.3±16.8	883.3±108.7	107.2±13.5	166.3±23.2	5.7±1.6
Plasma concentration at 1 hr (ng/mL)	181.3±8.4	291.3±45.5	301.7±12.7	305.3±63.3	58.1±9.6
Plasma concentration at 4 hr (ng/mL)	15.9±2.0	120.3±18.6	29.7±2.3	81.5±10.2	3.0±0.8
Heart/Plasma (H/P) ratio at 4 hr	8.9±1.9	7.6±2.2	3.6±0.2	2.1±0.3	1.9±0.2
Adjusted H/P ratio *	3.9±0.5	10.7±3.0	1.0±0.05	1.2±0.2	0.001±0.0003

	Evogliptin	**Saxagliptin**	**Teneligliptin**
Anti-calcifying EC50 (ng/mL)	179.8	172.2	544.1
Heart tissue concentration at 1 hr (ng/g)	1496.7±201.3	129.1±34.0	1346.7±70.9
Heart tissue concentration at 4 hr (ng/g)	397.0±27.9	14.8±4.3	262.0±39.0
Plasma concentration at 1 hr (ng/mL)	188.3±35.9	84.9±40.4	862.7±175.0
Plasma concentration at 4 hr (ng/mL)	33.6±2.8	3.3±2.0	138.7±39.6
Heart/Plasma (H/P) ratio at 4 hr	11.8±0.6	5.2±1.5	1.9±0.2
Adjusted H/P ratio *	26.1±1.3	0.4±0.1	0.9±0.1

EC50, half maximal effective concentration for anti-calcification. Values are presented as mean ± SD.

* Adjusted by absolute tissue concentration relative to the half maximal effective concentration for anti-calcification obtained from *in vitro* experiment

Supplemental Table 2. Baseline clinical characteristics before and after propensity score matching

	Before PS matching				After PS matching (1:2)		
	Favourable DPP-4 inhibitor non-user (N=184)	Favourable DPP-4 inhibitor user (N=28)	Overall P Value	SMD	Favourable DPP-4 inhibitor non-user (N=50)	Favourable DPP-4 inhibitor user (N=25)	SMD
Male, n (%)	97 (52.7)	14 (50.0)	0.789	-0.054	27 (50.0)	14 (50.0)	0.000
Age, years	71.6 ± 8.3	73.1 ± 7.4	0.438	0.164	72.5 ± 8.9	73.1 ± 7.4	0.077
Dyslipidemia, n (%)	188 (64.1)	19 (67.9)	0.701	-0.079	38 (70.4)	19 (67.9)	0.053
Coronary artery disease, n (%)	67 (36.4)	16 (57.1)	0.036	0.424	30 (60.0)	13 (52.0)	0.164
Baseline eGFR, ml/mim	67.3 ± 20.5	58.3 ± 15.4	0.026	0.496	59.2 ± 17.3	59.8 ± 15.2	0.038
Baseline AV mean pressure gradient, mmHg	18.4 ± 4.7	18.8 ± 5.6	0.724	0.068	18.9 ± 5.2	18.9 ± 5.6	-0.023
Follow-up duration, years	5.3 ± 1.9	4.4 ± 1.9	0.021	-0.477	4.6 ± 1.8	4.4 ± 1.9	-0.116
Use of statin, n (%)	130 (70.6)	23 (82.1)	0.206	-0.273	46 (85.2)	23 (82.1)	0.072
Use of metformin, n (%)	143 (77.7)	19 (67.9)	0.252	0.223	37 (68.5)	19 (67.9)	0.015
Use of sulfonylurea, n (%)	103 (56.0)	23 (82.1)	0.009	0.589	43 (86.0)	20 (80.0)	0.135
Use of insulin, n (%)	55 (29.9)	9 (32.1)	0.809	-0.049	15 (27.8)	9 (32.1)	-0.094

1) Discrimination: C-statistics = 0.776

2) Calibration: Hosmer-Lemeshow statistics's p-value = 0.5053

Supplemental Table 3. Hemodynamic progression of aortic stenosis in patients with absence or presence of DPP-4 inhibitor

	Non user (n=115)	DPP-4 inhibitor user (n=97)	Overall P value	Adjusted P value*
Annual change of AV maximal velocity, cm/s/year	17.7 ± 17.1	14.3 ± 16.0	0.143	0.366
Annual change of mean pressure gradient, mmHg/year	3.2 ± 3.2	2.3 ± 2.8	0.042	0.177
Annual change of peak pressure gradient, mmHg/year	5.2 ± 5.3	4.1 ± 4.9	0.123	0.330

*Adjusted for age, sex, and use of statin, metformin and insulin

AV, aortic valve; DPP-4, dipeptidyl peptidase-4

Supplemental Table 4. Post-hoc analyses for the comparison of annualized aortic stenosis progression rates according to the medications

Combinations	Difference of Vmax			Adjusted difference*		
	Means	95% CI	p-value	Means	95% CI	p-value
Favourable DPP-4 inhibitor user and non-user	-9.7	-(17.8-1.6)	0.014	-8.9	-(16.2-0.8)	0.028
Favourable and unfavourableDPP-4 inhibitor user	-8.9	-(17.5-0.3)	0.039	-9.4	-(17.9-1.1)	0.030
Unfavourable DPP-4 inhibitor user and non-user	-0.8	-(6.6--5.1)	0.950	0.6	-(4.6--7.8)	0.960
Combinations	**Difference of mean pressure gradient**			**Adjusted difference***		
	Means	**95% CI**	**p-value**	**Means**	**95% CI**	**p-value**
Favourable DPP-4 inhibitor user and non-user	-1.9	-(3.3-0.4)	0.011	-1.8	-(3.3-0.2)	0.010
Favourable and unfavourableDPP-4 inhibitor user	-1.4	-(3.0--0.2)	0.100	-1.6	-(3.2--0.1)	0.049
Unfavourable DPP-4 inhibitor user and non-user	-0.5	-(1.5--0.6)	0.581	-0.2	-(1.3--0.9)	0.926
Combinations	**Difference of peak pressure gradient**			**Adjusted difference***		
	Means	**95% CI**	**p-value**	**Means**	**95% CI**	**p-value**
Favourable DPP-4 inhibitor user and non-user	-2.9	-(5.4-0.4)	0.021	-2.3	-(5.4-0.2)	0.028
Favourable and unfavourableDPP-4 inhibitor user	-2.5	-(5.2--0.2)	0.072	-2.8	-(5.5-0.0)	0.047

Unfavourable DPP-4 inhibitor user and non-user	-0.4	-(2.2--1.4)	0.881	0.0	-(1.6--2.0)	1.000

CI, confidence interval; Vmax, maximal transaortic valve jet velocity

* adjusted for age, sex, coronary artery disease, use of statin, baseline estimated glomerular filtration rate and use of metformin, sulfonylurea, and insulin

Supplemental Table 5. Changes of echocardiographic parameters after propensity matching

	Favourable DPP-4 inhibitor non-user (n=50) Means ± standard deviation (95% confidence limits)	**Favourable DPP-4 inhibitor user (n=25)** Means ± standard deviation (95% confidence limits)	**P-value**
Annual change of maximal velocity	19.0 ± 17.1 20.5 (13.7 – 27.3)	8.1 ± 12.9 9.2 (2.7 – 15.7)	0.001* 0.002^
Annual change of peak pressure gradient	5.5 ± 5.4 6.0 (4.0 – 8.1)	2.4 ± 4.1 2.9 (0.7 – 4.1)	0.013* 0.006^
Annual change of mean pressure gradient	3.3 ± 3.5 3.5 (2.2 – 4.9)	1.5 ± 3.1 1.9 (0.4 – 3.3)	0.031* 0.047^

*mixed model matched pair; ^mixed model matched pair with adjustment of coronary artery disease, baseline mean pressure gradient, follow-up duration and use of sulfonylurea