

Development of DPP-IV Inhibitors

A Therapeutic Mechanism for the Treatment of Type II Diabetes

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Introduction:

Dipeptidyl peptidase-IV (DPP-IV) inhibitors are currently being developed as potential drugs for the treatment of type 2 diabetes. Initial observation of glucagon-like peptide-1 (GLP-1) being rapidly cleaved and inactivated by the protease DPP-IV formed the basis for design and development of novel inhibitors of DPP-IV. Inhibition of DPP-IV proteolytic activity is shown to increase the levels of GLP-1 and prolong the action of GLP-1, which is released postprandially from the L-cells in the gut and increases insulin secretion (the incretin concept), resulting in improved glucose tolerance¹. Encouraging preclinical and clinical results of DPP-IV inhibitors by several companies including NVP-LAF237 (Novartis)², and MK-0431 (Merck)³ have provided proof to initial observations. MK-0431 (Sitagliptin) is approved for treatment of type 2 diabetes⁴.

Here we present in vitro and in vivo profiling of novel, orally active, potent and selective small molecule DPP IV inhibitors under development for treatment of type -2 diabetes.

Methods:

1. Enzyme Assay

DPP-IV, DPP-8 and DPP-9 inhibition measurement in vitro - DPP-IV activity was measured using a fluorometric assay in which the liberation of AMC from the substrate Gly-Pro-AMC was monitored. DPP-IV enzyme isolated from porcine kidney and the substrate Gly-Pro-AMC were purchased from Sigma Chemical Company, USA. rDPP IV, rDPP-8 and rDPP-9 (human) were expressed in Baculo virus, purified and used for assay. The assay was carried out in the buffer 20 mM Tris.HCl, pH 8.0. Test compounds were incubated with the enzyme in a 96-well black flat bottom microtiter plate for 20 min at 30⁰ C and the reaction was initiated by the addition of 50 μM Gly-Pro-AMC. For DPP-9 inhibition assay, buffer contained 0.1% BSA. After 30 min incubation at 30⁰ C, the fluorescence was measured. The excitation and emission wavelengths were 365 and 465 nm respectively.

DPP-IV inhibition measurement exVivo – Rat and human plasma were used for the assay using same protocol as described above. 2 μl rat/human plasma were used for every reaction. Total assay volume was 100 μl.

2. General ADME assays

Microsomal metabolic stability

Rat liver microsomes were incubated with test compounds (10 μ M in 0.2% DMSO) at 37°C for 30 minutes. The reaction was stopped by the addition of acetonitrile containing haloperidol as internal standard. Precipitated protein was removed by centrifugation and the supernatants were analyzed by HPLC-UV/VIS or LC/MS method. Stability was assessed by the disappearance of compound based on the change in analyte to internal standard peak height ratio. Metabolic stability was defined as the amount of substrate metabolized by the incubation with rat liver microsomes and expressed as a percentage of the initial amount of substrate (%S).

Permeability across MDCK cell monolayer

Madin-Darby Canine Kidney cells (ATCC) were grown in DMEM supplemented with 10% fetal bovine serum in 24-well plates loaded with polycarbonate Millicell inserts (Millipore, 12 mm diameter, 0.4 μ m, 50,000 cells/insert). Cells were grown for 3 days and monolayer integrity was assessed by measuring TEER (Trans-Epithelial resistance). Drugs were applied at 50 μ M in Hank's buffered salt solution containing 0.5% DMSO to the apical chamber and the transport assay was carried out for 2 hours at 37°C. The appearance of compound in the basal chamber was determined by HPLC-UV and compounds were ranked based on their apparent permeability (P_{app}).

3. Pharmacokinetic studies

Oral and intravenous pharmacokinetic studies were conducted in male Wistar rats (200-250 g). Overnight fasting of animals was carried out for conducting oral pharmacokinetic study. Blood (0.3 ml at each time point) was collected by retro orbital bleeding under light anesthesia before administration of test formulation (0 min). 5 animals per route were administered test substance (5 mg/kg) dissolved in vehicle Normal Saline given at 1 ml/kg. Additional blood samples were drawn at 0.25, 0.5, 1, 2, 4, 6, 8 hours after administration of test formulation in EDTA tubes. Plasma was separated and stored at -80° C until analysis.

Oral and intravenous pharmacokinetic studies were conducted in male Beagle dogs. Overnight fasting of animals was carried out for conducting oral pharmacokinetic study. Blood was collected before administration of test formulation (0 min). 5 animals per route were administered test substance (3 mg/kg) dissolved in vehicle Normal Saline given at 1 ml/kg. Additional blood samples were drawn at 0.5, 1, 2, 3, 5, 8, 10 and 24 hours after administration of test formulation in EDTA tubes. Plasma was separated and stored at -80° C until analysis.

Determination of AU compounds in Plasma: Plasma samples were subjected to liquid-liquid extraction with Ethyl Acetate. Ethyl acetate containing AU compounds were evaporated to dryness under steam of Nitrogen and reconstituted with Acetonitrile and Water (1:1). Samples were analysed by LC/MS/MS with atmospheric pressure chemical ionization (APCI). Concentrations were determined based on a standard curve ranging from 0 to 2000 ng/ml. The limit of quantitation was 1 ng/ml.

DPP IV inhibition in rat plasma from pharmacokinetic studies: 5 μ L of rat/dog plasma collected during oral pharmacokinetic studies was used for estimation of DPP IV activity by

fluorometric assay. DPP IV activity after administration of test formulation was normalized to 0 minute activity in plasma.

4. Efficacy in animal models of Diabetes

Glucose Tolerance test in *db/db* mice and Zucker *fa/fa* rats: Five- to six- week old healthy male *db/db* mice were obtained from Jax laboratories, USA, Eight- to nine- week old healthy male Zucker *fa/fa* rats were obtained from Charles River laboratories, USA, and acclimatized for a week before the initiation of the study. The animals were randomized based on their baseline blood glucose level into three groups ($n=6$ each) to investigate the effect of treatment during oral glucose tolerance test (OGTT). For OGTT, animals were fasted for 16-18 h. Sixty minutes before glucose load of 1.5g/kg, animals were administered respective treatment orally. Blood glucose concentrations were measured at 15, 30, 60 and 120 minutes. The reduction in blood glucose produced by the compounds was calculated using the area under the curve method with respect to basal values. GraphPad Prism® software was used for statistical analysis.

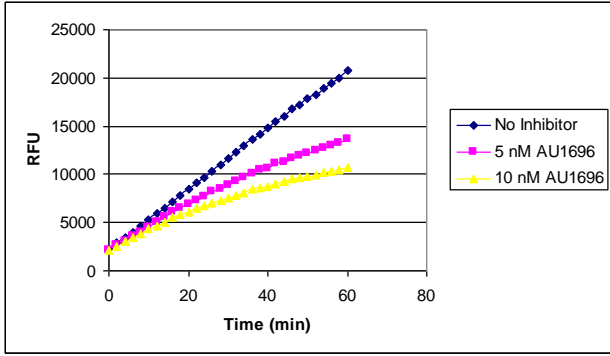
Results:

In vitro profile:

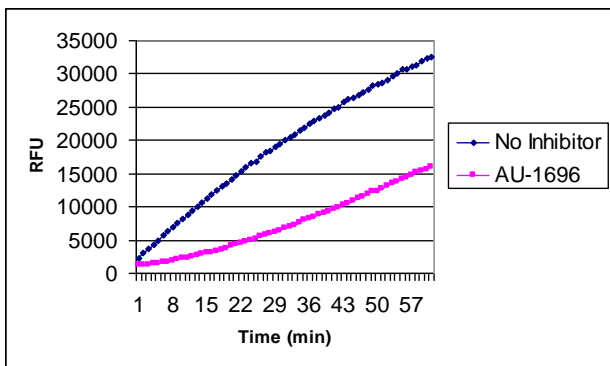
Assay	IC 50 (nM)		
	AU 1696	LAF- 237	MK-0431
Porcine DPP IV	4.7	61	31
rDPP IV (Human)	1.8	40.5	19.5
Human plasma	3.5	51	25.5
Rat plasma	2.8	35	15
DPP 8* (Selectivity)	541 (300)	12000 (296)	57500 (2950)
DPP 9* (Selectivity)	44 (25)	89 (3.2)	75000 (3850)
Binding kinetics	Slow binding	Slow binding	Slow binding
Enzyme kinetics	reversible	reversible	reversible

* compared to rDPP IV (human)

Table 1: AU 1696 is a potent, slow binding, reversible DPP IV inhibitor with moderate specificity against DPP 8 and DPP 9.



(A)



(B)

Figure 1: Time dependent inhibition of DPP-IV activity (A) and reversible binding of AU 1696

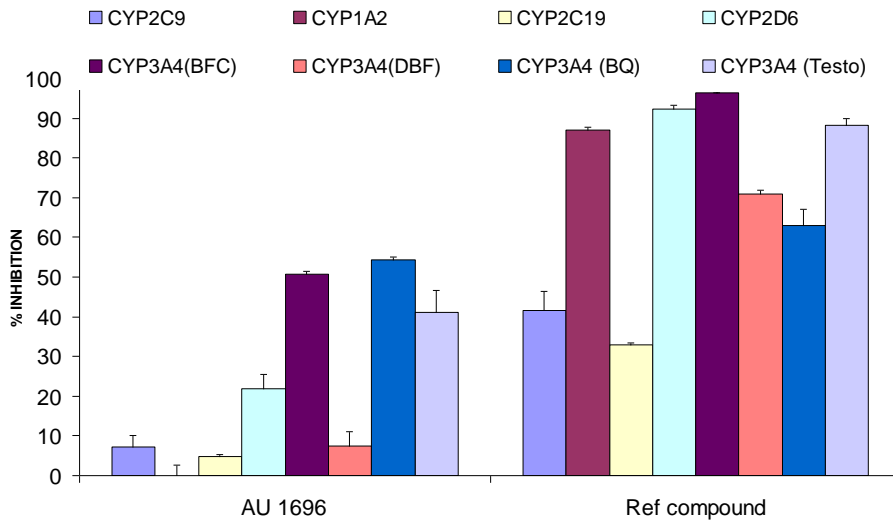


Figure 1: AU 1696 at 10 μ M produced no significant inhibition of the CYP450 enzyme tested and has a low potential for drug-drug interaction

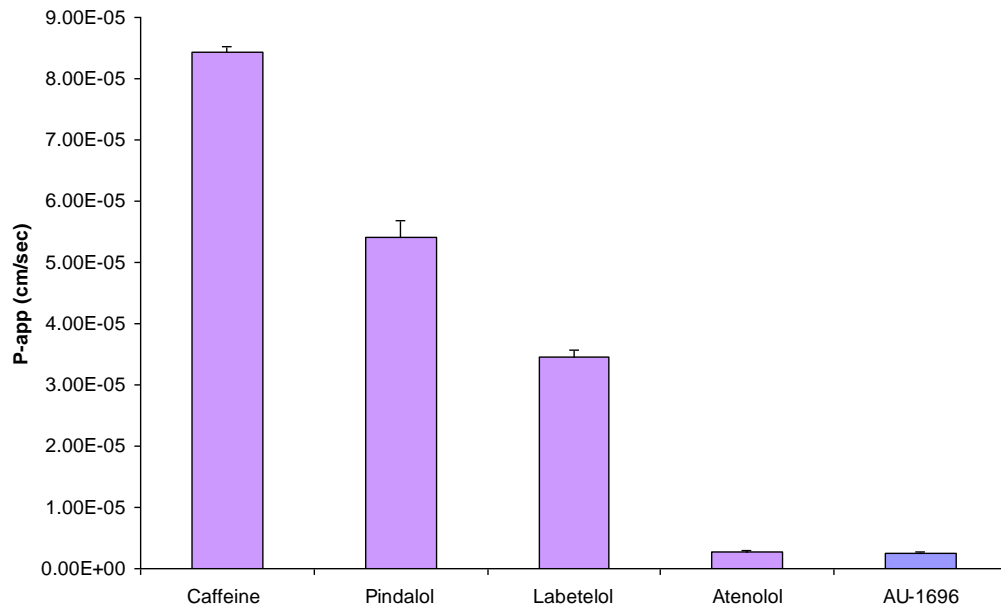


Figure 2: AU 1696 is low permeable through MDCK monolayer contributing for slow absorption and long exposure.

In vivo studies:

Parameter	Units	AU 1696		LAF 237		MK 0431
		Rat	Dog	Rat	Monkey*	Rat
N		5	3	5	-	4
Dose	mg/kg b.w	5	3	5	0.3	5
C _{max}	ng/ml	157.68	479.5	86.88	88.89	308.70
AUC _{0-inf}	ng.h. ml ⁻¹	332.7	1808.08	228.25	-	1690.50
T _{max}	hr	0.5	0.75	0.7	1.15	1.25
T _{1/2}	hr	2.64	1.89	2	1.5	3.54
V _d	l/kg	66.91	3.68	56.16	0.7	13.78
CL	l/h/kg	15.84	1.66	22.7	1.5	3.11
Terminal t _{1/2}	hr	2.76	1.51	1.71	-	2.76
F	%	28.4	75	51.8	>90%	69.64

* Published values²

Table 2: Single dose oral pharmacokinetics of AU 1696 in rats and dogs

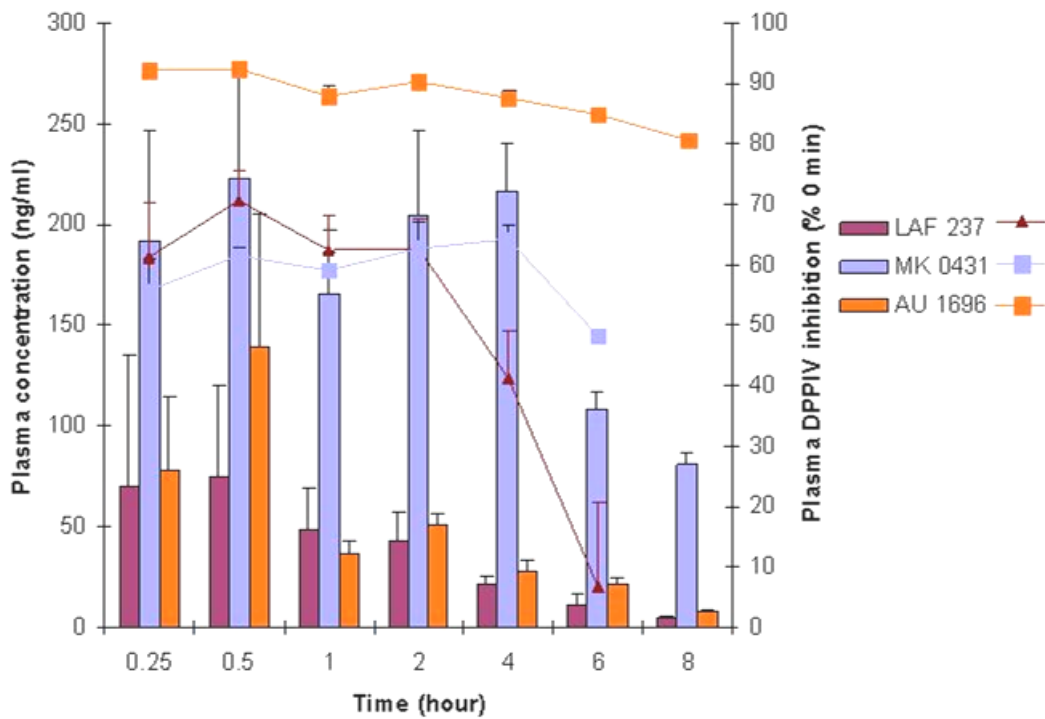


Figure 3. Correlation of plasma levels of AU 1696 with DPP-IV inhibition after oral administration in overnight fasted Wistar rats (n=5) at 5 mg/kg
AU 1696 produces greater than 80% inhibition of DPP-IV which is sustained up to 8 hours after compound administration. There is a good correlation between plasma levels of AU 1696 and inhibition of DPP-IV.

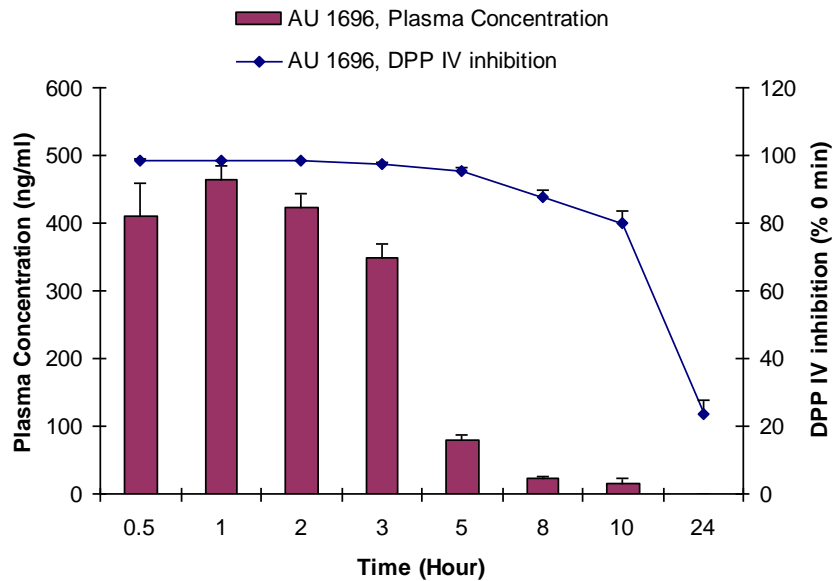


Figure 4: Correlation of plasma levels of AU 1696 with DPP-IV inhibition after oral administration in overnight fasted Beagle dogs (n=3) at 3 mg/kg
AU 1696 produces greater than 90% inhibition of DPP-IV which is sustained up to 10 hours after compound administration. There is a good correlation between plasma levels of AU 1696 and inhibition of DPP-IV.

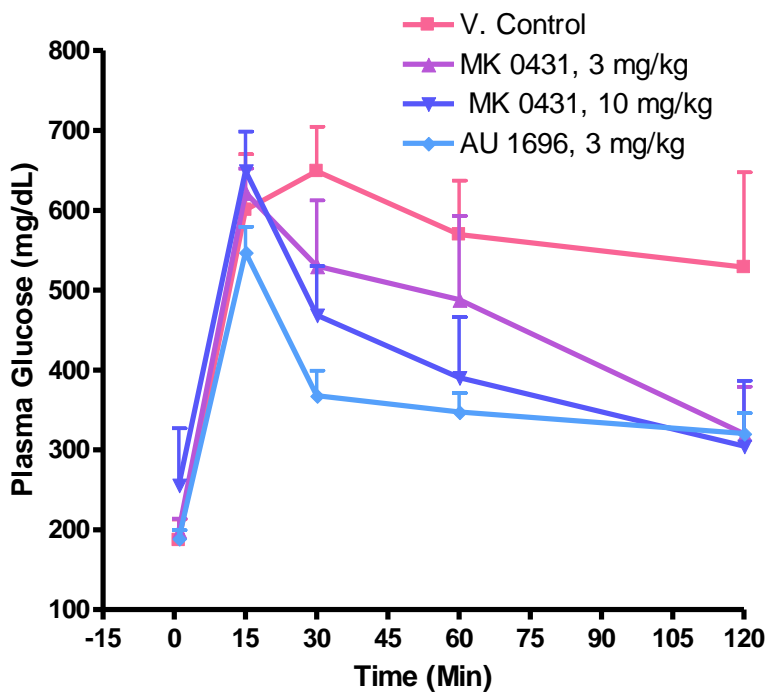


Figure 5: Effect of AU 1696 on glucose excursion in male db/db mice. Test compound administration 60 min before glucose challenge.

Comparison of AUC (0-120 min) db/db mice				
Parameter	V. Control	MK 0431, 3 mg/kg	MK 0431, 10 mg/kg	AU 1696, 3 mg/kg
Baseline	186.9	198.9	256	189.1
Total Area	44098	30465	18204	20463
% Reduction		30.92	58.72	53.60

Table 3: AU 1696 produced a significant reduction in area under the curve of glucose excursion as compared to vehicle treated animals.

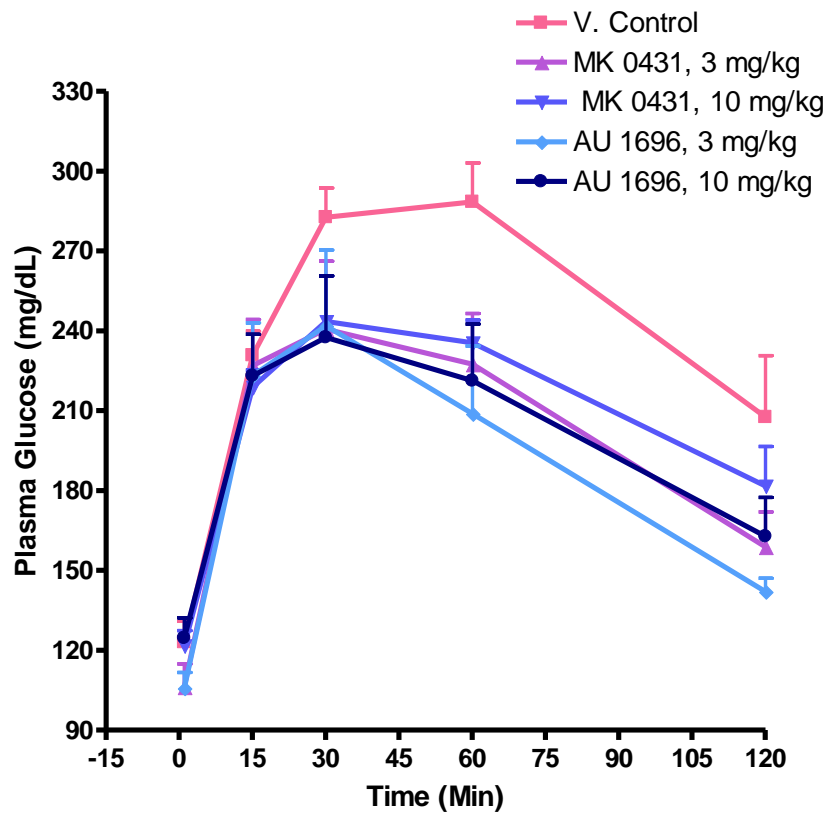


Figure 6: Effect of AU 1696 on glucose excursion in male Zucker fa/fa rats. Test compound administration 60 min before glucose challenge.

Comparison of AUC (0-120 min) Zucker fa/fa rats					
Parameter	V. Control	MK 0431, 3 mg/kg	MK 0431, 10 mg/kg	AU 1696, 3 mg/kg	AU 1696, 10 mg/kg
Baseline	123	106.2	121.7	105.8	124.6
Total Area	15196	11875	11118	10547	9515
% Reduction		21.85	26.84	30.59	37.38

Table 4: AU 1696 produced a significant reduction in area under the curve of glucose excursion as compared to vehicle treated animals.

Cytochrome P-450 Enzyme Inhibition

AU 1696 at 10 μ M produced no significant inhibition of the CYP450 enzyme tested and has a low potential for drug-drug interaction.

AU 1696 produced no significant effect on the HERG K⁺ channel at 10 μ M.

No adverse hematological, biochemical and histopathological changes in 14 day repeated dose oral toxicity of AU 1696 (10, 30, 100 mg/kg) in wistar rats

Conclusion:

1. AU 1696 is a slow binding, reversible inhibitor of DPP IV with moderate specificity against DPP 8 and DPP 9.
2. AU 1696 has good pharmacokinetic properties with long lasting *in vivo* DPP IV inhibition. Better formulation can improve *in vivo* exposure.
3. AU 1696 is highly efficacious in diabetic animal models.
4. AU 1696 has no safety concerns till 100 mg/kg by repeated oral administration.

References:

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4. <http://www.fda.gov/bbs/topics/NEWS/2006/NEW01492.html>