Inhibition of Dipeptidyl-Peptidase IV Does Not Increase Circulating IGF-1 Concentrations in Growing Pigs

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The enzyme dipeptidyl peptidase-IV (DPP-IV) inactivates a variety of bioactive peptides, including glucagon-like peptide-1 (GLP-1) and growth hormone releasing hormone (GHRH). Inhibiting DPP-IV in order to increase circulating GLP-1 is of interest as a treatment for Type II diabetes. Inactivation of DPP-IV may also increase circulating GHRH, potentially enhancing growth in domestic animals. To test the hypothesis that inhibition of DPP-IV activity will influence the growth hormone/IGF-1 axis, growing pigs (*Sus scrofa domesticus*, 78 kg) were treated with a DPP-IV inhibitor (Compound 1, the 2,5-difluorophenyl analog of the triazolopiperazine MK0431, sitagliptin), and plasma concentrations of IGF-1 were monitored. Pigs were administered either sterile saline (0.11 ml/kg followed by a continuous infusion at 2 ml/hr for 72 hrs, controls, $n = 2$), Compound 1 (2.78 mg/kg followed by a continuous infusion at 0.327 mg/kg·hr for 72 hrs, $n = 4$) or GHRH (0.11 ml/kg sterile saline, followed by a continuous infusion of GHRH at 2.5 μg/kg·hr for 48 hrs, $n = 4$). Plasma concentrations of Compound 1 were maintained at 1 μM, which resulted in a 90% inhibition of circulating DPP-IV activity. Relative to the predose 24-hr period, area under the IGF-1 concentration curve (AUC) tended to be lower ($P = 0.062$) with Compound 1 (−79 ± 130 ng/ml·hr) than controls (543 ± 330 ng/ml·hr). GHRH treatment increased the IGF-1 AUC (1210 ± 160 ng/ml·hr, $P = 0.049$ vs. controls and $P = 0.001$ vs. Compound 1). We conclude that inhibition of DPP-IV does not alter the circulating levels of IGF-1 in the growing pig.
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Key words: growth hormone releasing hormone; DPP-IV; sitagliptin analog

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Growth hormone (GH) secretion from the pituitary gland is regulated by the stimulatory activity of growth hormone releasing hormone (GHRH) and the inhibitory activity of somatostatin (1, 2). Circulating GH concentrations in turn stimulate secretion of insulin-like growth factor 1 (IGF-1) (3). Administration of either exogenous GHRH or GH leads to an increase in IGF-1, enhanced growth performance, and decreased fat deposition in swine (4–6).

GHRH belongs to a protein superfamily that includes glucagon, glucagon-like peptides (GLPs), secretin, and other bioactive peptides (7). GHRH shares an α-helical structure (7) and susceptibility (8) to inactivation by dipeptidyl peptidase IV (DPP-IV) with many other family members (e.g., GLP-1, gastric-inhibiting peptides [GIPs], and peptide histidine methionine). Indeed, inhibition of DPP-IV increases the circulating half-life of the incretin hormones GLP-1 and GIP, improving glucose tolerance in Type II diabetics (9). Complete inhibition of DPP-IV does not appear to be necessary: 2- to 3-fold increases in plasma concentrations of GLP-1 were achieved in mice with inactivation of 84% to 96% of plasma DPP-IV (17). Thus, there has been much interest in the pharmaceutical industry in developing DPP-IV inhibitors for the treatment of Type II diabetes (10, 11).

DPP-IV exists as both a membrane-spanning form present in cells throughout the body and a soluble circulating form. Both forms of DPP-IV have identical enzymatic activity (9) and cleave a wide range of bioactive peptides *in vitro*, including hormones, neuropeptides, and chemokines (12). One potential regulatory role of DPP-IV is the inactivation of GHRH through cleavage of the active form, GHRH(1–44)-NH_2, to the N-terminally shortened inactive form, GHRH(3–44)-NH_2, (13). While trypsin-like degradation of GHRH also occurs, *in vitro* studies using GHRH analogs designed to resist cleavage at the N-terminus have demonstrated that the primary degradation of GHRH is via DPP-IV (13, 14). Substitution of Ala2 with DAla prevents DPP-IV proteolysis (13, 15), and admin-

istration of this analog increases GH release in swine up to 2-fold (7). The His1, Val2 analog of GHRH is also not degraded by DPP-IV *in vitro*, and it demonstrates increased plasma stability over native GHRH (16). GHRH analogs containing the His1, Val2 substitutions were 5.4- to 12.5-fold more potent than native GHRH in release of GH in swine (16). Thus, inhibition of DPP-IV *in vivo* may increase endogenous concentrations of GHRH and enhance GH secretion.

One of the concerns surrounding DPP-IV inhibition therapies for Type II diabetes in humans is, that in addition to stabilizing the target incretins GLP-1 and GIP, there is potential for stabilization of multiple bioactive peptide substrates such as GHRH. Increasing circulating active GHRH would stimulate the GH/IGF-1 axis, resulting in increased IGF-1 concentration. While studies have shown beneficial effects of exogenous administration of GHRH (17, 18) and GH (19) in humans, elevation of IGF-1 in diabetics would not be desired, given its potential to exacerbate insulin resistance (20).

The present study was designed using IGF-1 plasma concentrations in pigs as a biomarker for the physiological relevance of DPP-IV activity in GHRH degradation. Domestic swine approaching market weight were dosed with a DPP-IV inhibitor to evaluate the potential for growth enhancement and/or adverse effects as a result of stabilization of endogenous GHRH.

Materials and Methods

Animals, Housing, and Surgical Procedures. Ten male castrate, crossbred swine (Stonehurst Farms, Strasburg, PA) with a pretrial mean weight of 78 ± 1.1 kg were used. Pigs were fed an 18% crude protein, 5% fat corn-soybean–based meal (F.M. Brown's Sons Inc., Birdsboro, PA) and housed individually in 1.4 × 1.4-m pens in an environmentally controlled facility. Water was available at all times via a nipple drinker system. All procedures were approved by the Merck Research Laboratories Institutional Animal Care and Use Committee.

Pigs were surgically fitted with double-lumen jugular catheters (Micro-Renathane, 0.2-cm o.d. × 0.1-cm i.d.; Braintree Scientific, Braintree, MA) approximately 14 days before the study. Anesthesia was induced with a cocktail containing Telazol (2 mg/kg, tiletamine HCl and zolazepam; Fort Dodge Laboratories, Fort Dodge, IA) and xylazine (5 mg/kg; Phoenix Scientific, St. Joseph, MO). Pigs were then endotracheally intubated, and anesthesia was maintained with 2% to 3% isoflurane (Anaquest, Liberty Corner, NJ) in oxygen with a flow rate of approximately 1 liter per min. Catheters were tunneled subcutaneously to a 5 × 5-cm subcutaneous pocket formed approximately 10 cm ventral to the dorsal midline and 10 cm caudal to the ear. Catheters were then connected to vascular access ports (Access Technologies, Skokie, IL). Patency of ports and catheters was maintained with a saline flush solution containing 50 U/ml heparin (Elkins-Sinn, Cherry Hill, NJ) and 1000 U/ml penicillin G (Pfizer Roerig Division, New York, NY). Pigs were treated with a prophylactic antibiotic (oxytetracycline, 22 mg/kg; Butler Co., Columbus, OH) and an analgesic (flunixin meglumine, 0.5 mg/kg; Fort Dodge Animal Health, Fort Dodge, IA).

Test Compounds. Compound 1 (7-[(3R)-3-amino-1-oxo-4-(2,5-difluorophenyl)butyl]-5,6,7,8-tetrahydro-3-(trifluoromethyl)-1,2,4-triazolo[4,3-a]pyrazine L-tartaric acid salt; Process Research, Merck Research Laboratories, Rahway, NJ), a small molecule inhibitor of DPP-IV, is the 2,5-difluorophenyl analog of the triazolopiperazine MK0431 (sitagliptin) (21). Compound 1 was dissolved in sterile saline (25 mg/ml). Growth hormone releasing factor amide (GHRH, fragment 1–29; Sigma-Aldrich, St. Louis, MO) was first dissolved in 0.1 *N* HCl and then normalized with 0.1 *N* NaOH for a final concentration of 5 mg/ml.

Experimental Design. Pigs were randomly assigned to one of three different treatment groups. All treatments were administered iv with a primed, continuous infusion delivered using Baxter APII Ambulatory Pumps (Baxter Healthcare Corp., Round Lake, IL). Controls ($n = 2$) were administered sterile saline consisting of a bolus infusion of 0.11 ml/kg followed by a continuous infusion at 2 ml/hr for 72 hrs. Compound 1 ($n = 4$) was administered in a bolus infusion at a dosage of 2.78 mg/kg followed by a continuous infusion at a dosage of 0.327 mg/kg·hr for 72 hrs. Animals dosed with GHRH ($n = 4$) were first administered a bolus infusion of 0.11 ml/kg sterile saline followed by a continuous infusion of GHRH at a dosage of 2.5 μg/kg·hr for 48 hrs and then a continuous infusion of sterile saline at 2 ml/hr for an additional 24 hrs.

Blood Sampling. Samples of blood (5 ml) were collected into tubes containing EDTA every 6 hrs beginning 24 hrs prior to the initiation of dosing. Blood collection and drug infusion were accomplished simultaneously using the dual-lumen catheters attached to individual subcutaneous access ports. The infusion side of the catheter was advanced approximately 2 cm further downstream in the jugular than the blood collection side. Harvested plasma was stored at –70°C until assayed.

Determination of Plasma Concentrations of Compound 1. Concentrations of Compound 1 were determined by high performance liquid chromatography tandem mass spectrometry using an ABI Sciex API 3000 mass spectrometer (Applied Biosystems, Foster City, CA) operated in positive ion atmospheric pressure chemical ionization mode with multiple-reaction monitoring. The high-performance liquid chromatography system interfaced to the mass spectrometer consisted of 2 Perkin-Elmer Series 200 micro pumps and a Perkin-Elmer Series 200 autosampler (Perkin-Elmer, Norwalk, CT). A volume of 0.05 ml plasma was spiked with 50 ng internal standard. Plasma was prepared for LC-MS/MS analysis by solid phase extraction using OASIS HLB extraction plates (30 mg; Waters Corp., Milford, MA). Plasma samples (diluted with 200 μl water)

were added to plates that had been preconditioned with methanol and then water (1 ml each), washed with water (1 ml), and eluted with methanol (1 ml). The organic eluent was concentrated to dryness under nitrogen at 350°C and reconstituted with 0.2 ml mobile phase. The reconstituted extracts were chromatographed using a Fluophase PFP column ((Thermo Hypersil-Keystone, Bellefont, PA) 50 × 2 mm, 5 micron) and eluted at 0.2 ml/min under isocratic conditions with acetonitrile–water (9:1) containing 5 m*M* ammonium formate/0.1% formic acid. Under these conditions Compound 1 eluted at 2.3 min. A standard curve was generated from the mean of two replicates that were made by spiking an equal volume of plasma from untreated animals with increasing amounts (0.05–4000 ng; 12 concentrations). The lower limit of quantification was 1 ng/ml.

Determination of Plasma DPP-IV Activity. The *in vitro* assay for measuring inhibition of plasma DPP-IV has been previously described (21). Briefly, plasma DPP-IV activity was measured using a continuous fluorometric assay with the substrate Gly-Pro-AMC, which is cleaved by DPP-IV to release the fluorescent AMC leaving group. The data are reported as percentage inhibition calculated as follows: %Inhibition = 100 $(1 - (V_t/V_c))$, where V_t is the rate of reaction of treated sample and V_c is the rate of reaction of control sample.

Determination of Plasma IGF-1. Analyses of plasma samples for IGF-1 concentrations were performed by the Endocrinology Laboratory at the Cornell University Animal Health Diagnostic Center (Ithaca, NY) using an immunoradiometric assay (DSL-5600 Active IRMA kit; Diagnostic Systems Laboratories, Webster, TX). The intra- and interassay coefficients of variation were 5.7% and 10.0%, respectively.

Data and Statistical Analysis. Area under the IGF-1 concentration curve (AUC) was calculated as increase over the predose 24 hrs and is reported as mean ± SE. Statistical analyses were performed using the General Linear Model of SAS (SAS Institute, Cary, NC).

Results

Preliminary studies demonstrated that in pigs Compound 1 has an *in vivo* half-life of 2.7 hrs and that plasma concentrations of 0.6 μ*M* result in DPP-IV inhibition of greater than 94% (data not shown). Plasma concentrations of pigs infused with Compound 1 in the current study are shown in Figure 1. The primed, continuous infusion resulted in Compound 1 concentrations at or above 1 μ*M* throughout the 72 hrs of infusion.

Selected reaction curves for Pig 1, typical of all assays, are shown in Figure 2. The rates of reaction at baseline and 72 hrs, calculated from the rates at the end of the 180-sec reactions, were 0.327 and 0.031 nmol/min, respectively. Inhibition at 72 hrs for Pig 1 was 91%. For all of the pigs, inhibition of DPP-IV by Compound 1 averaged 91% ± 1% over the 72-hr period (Fig. 3).

Inhibition of circulating DPP-IV did not result in a significant increase in circulating IGF-1 (Fig. 4). Relative to the predose 24-hr period, the AUC of IGF-1 following treatment with Compound 1 was −79 ± 130 ng/ml·hr, and the IGF-1 AUC from saline controls was 543 ± 330 ng/ml·hr ($P = 0.062$). In contrast, pigs treated with GHRH had a robust increase in IGF-1, with the AUC increased over the predose 24 hrs by 1210 ± 160 ng/ml·hr ($P = 0.049$ compared with saline controls and $P = 0.001$ compared with Compound 1–dosed pigs).

Discussion

In this study, inhibition of DPP-IV with a potent, selective (21) inhibitor did not result in increased circulating IGF-1 in pigs. This result indicates that DPP-IV inhibition does not significantly modulate the GH/IGF-1 axis in these animals, and, hence, precludes DPP-IV inhibition as a growth-enhancing strategy in production animals. Conversely, these data ameliorate concerns that inhibition of DPP-IV as a therapy for Type II diabetes may elevate circulating IGF-1 levels and adversely affect the insulin sensitivity of the individual.

There are several possible explanations for why there is no change in circulating IGF-1 levels following DPP-IV inhibition. While enzymatic degradation of GHRH by DPP-IV is efficient *in vitro*, it is not the only pathway of GHRH metabolism *in vivo*. For example, GHRH can be inactivated by enzymatic cleavage at Arg11, Lys12, Arg20, and Lys21 (22). Furthermore, inhibition of DPP-IV would not prevent inactivation of GHRH by oxidation of methionine (Met27 conversion to Met(0)27) (16). In addition, the rate of GHRH degradation has been reported to differ across tissues. GHRH(1–29)NH_2 is degraded more rapidly in liver homogenates (4) but at a slower rate in pituitary and hypothalamus preparations (23); when compared to serum, however, the major cleavage in theses tissues is not DPP-IV dependant.

Although administration of Compound 1 to the animals in this study caused essentially complete inhibition of soluble DPP-IV activity in plasma, that inhibition may not have prevented the membrane-bound enzyme from degrading GHRH *in vivo*. While the activity of the circulating form of DPP-IV can be successfully blocked *in vitro* (13), DPP-IV activity in several tissues can be orders of magnitude greater than that in serum (24). For instance, serum DPP-IV activity with Gly-Pro-4-nitroanilide as a substrate was reported to be approximately 10 nmol/min·mg, whereas liver activity (1.6×10^3 nmol/min·mg) and kidney activity (1.5×10^4 nmol/min·mg) were considerably higher (24). In contrast, there is evidence the plasma DPP-IV inhibition is a good surrogate for inhibition of the membrane-bound form *in vivo*. Inhibition of plasma DPP-IV activity in preclinical species and in man at a similar percentage as we obtained in

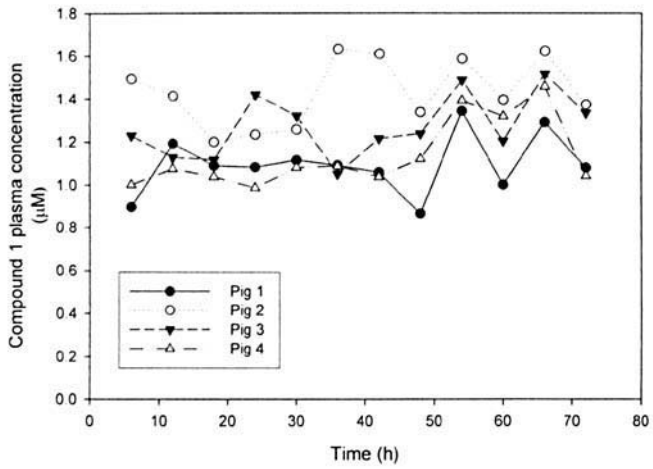

Figure 1. Plasma concentrations of Compound 1 from individual pigs dosed at Time 0 with a bolus of 2.78 mg/kg followed by a continuous infusion of 0.327 mg/kg·hr for 72 hrs.

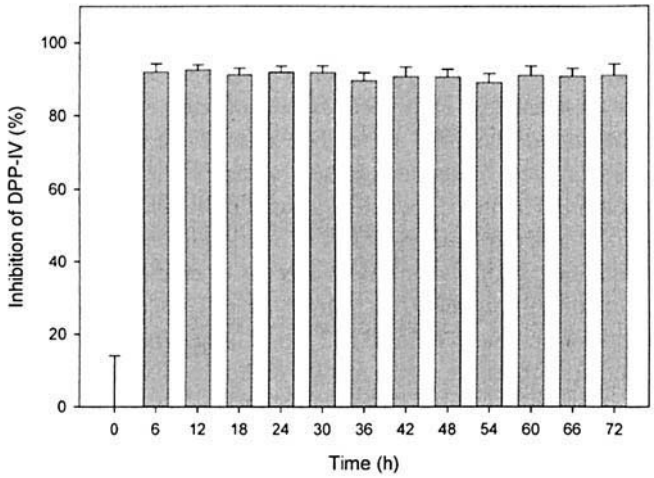

Figure 3. Inhibition of DPP-IV (mean ± SE) in plasma obtained from pigs ($n=4$) dosed with a bolus of Compound 1 (2.78 mg/kg), followed by a continuous infusion of 0.327 mg/kg·hr for 72 hrs. Baseline DPP-IV activity for Pig 1 was 59 nmol/min. (The percentage of inhibition as determined using the *in vitro* assay underestimates the percentage of inhibition achieved *in vivo*, as Compound 1 is a competitive, rapidly reversible inhibitor, and assay of plasma DPP-IV activity requires: (1) dilution of plasma, which results in a dilution of the total inhibitor; and (2) presence of substrate that competes with inhibitor for binding to the enzyme.)

the pig is correlated with stabilization of GLP-1 and a subsequent increase in circulating levels (25, 26).

In addition, GHRH is an excellent substrate for DPP-IV, with a rate constant at physiologic concentrations that is several times greater than that of GLP-1 or GIP (24). Therefore, it is possible that a small percentage of DPP-IV remains uninhibited and could metabolize sufficient amounts of GHRH to prevent a physiologic shift in the GH axis. GHRH homologous desensitization is another possible mechanism controlling GH secretion and, thus, IGF-1 secretion. An increase in GHRH may act to decrease the number of GHRH receptors in the pituitary. Incubation of primary rat pituitary cells with GHRH, but not GH, decreased the mRNA of GHRH receptors (27). However, GH release in primary rat pituitary cell cultures (27) was significantly stimulated by as little as 0.01 n*M* (50 pg/ml) GHRH, whereas a 10-fold increase in GHRH (0.1 n*M*) was necessary to significantly reduce receptor mRNA levels.

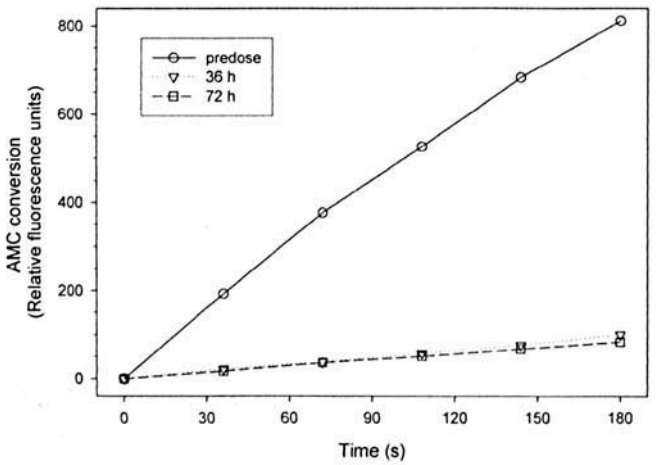

Figure 2. Typical DPP-IV reaction curves in plasma obtained from Pig 1, which was dosed with a bolus of Compound 1 (2.78 mg/kg), followed by a continuous infusion of 0.327 mg/kg·hr for 72 hrs.

Furthermore, in the current study, infusion of GHRH *in vivo* led to an increase in circulating IGF-1. These data suggest that increases in circulating GHRH may not induce downregulation of GHRH receptors; however, receptor mRNA upregulation is present following immunoneutralization of GHRH in rats (28). Therefore, GHRH may contribute to regulation of the GH axis through upregulation, but not downregulation of receptor expression.

To assess the question of whether DPP-IV plays a physiologic role in the metabolism of a particular peptide, four criteria need to be assessed (9). The first criterion, *in*

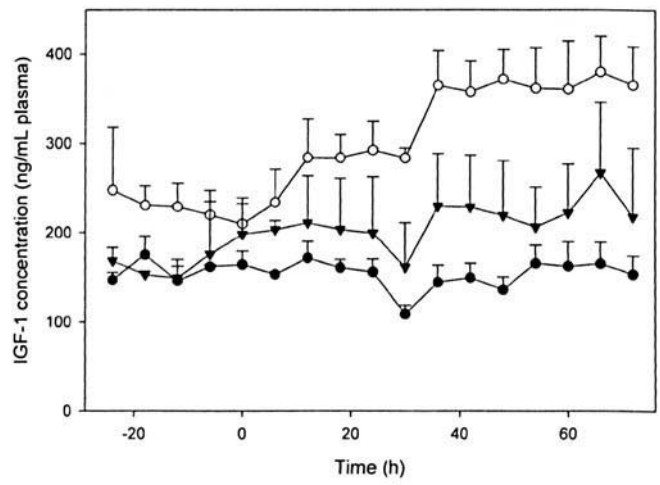

Figure 4. IGF-1 plasma concentrations (mean ± SE) in pigs infused with saline (bolus of 0.11 ml/kg, continuous infusion of 2 ml/hr; $n=2$; ▼), growth hormone releasing factor (bolus of 0.11 ml/kg saline, continuous infusion of GHRH at 2.5 μg/kg·hr for 48 hrs, then saline at 2 ml/hr; $n=4$; ○), or Compound 1 (bolus of 2.78 mg/kg, continuous infusion of 0.327 mg/kg·hr for 72 hrs; $n=4$; •). Dosing began at Time 0.

vitro cleavage by DPP-IV, is met in the cases of GLP-1, GIP, and GHRH, as well as various other endogenous bioactive peptides. The second, successful inhibition of degradation by DPP-IV inhibitors *in vitro*, is also met for GLP-1, GIP, and GHRH. GHRH degradation is decreased in plasma incubations with DPP-IV inhibitors (14), and the plasma stability of GHRH analogs that are resistant to DPP-IV is greater than that of native GHRH (16). The third criterion, an increase in the relative proportions of intact to degraded endogenous peptide following *in vivo* treatment with a DPP-IV inhibitor, has been met by GLP-1 and GIP but has not been demonstrated for GHRH and was not directly measured in the current study. The final criterion is demonstration of a physiologic change in animals that lack expression of functional DPP-IV. This criterion has been met for GLP-1 in the Japanese Fischer 344 rat and the $CD26^{-/-}$ C57/B6 mouse, which are DPP-IV negative and have increased circulating GLP-1 and improved glucose tolerance (29, 30). There is no indication of enhanced GH secretion in the DPP-IV–negative rodents, as the Japanese Fischer 344 rats grow at the same rate as DPP-IV–positive Fischer 344 rats (31), and the male $CD26^{-/-}$ mice are slightly smaller than wild-type mice (29, 30).

We conclude that the failure to induce increases in IGF-1 concentrations in pigs with inhibition of DPP-IV activity, as seen in the current study, and the normal (or slightly slower) growth rate of DPP-IV–negative rodents suggest that DPP-IV is not a major regulator of endogenous GHRH activity nor, by extension, the GH/IGF-1 axis.

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