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Identification of a novel metabolite of vildagliptin in humans: Cysteine targets the nitrile moiety to form a thiazoline ring

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ABSTRACT

The dipeptidyl peptidase-4 (DPP-4) inhibitor vildagliptin (VG) is used to treat type 2 diabetes. In rare cases, VG-induced liver injury has been reported. One case report suggested that immune responses were involved in the hepatotoxicity. However, the underlying mechanisms of VG-induced hepatotoxicity are uncertain. In the present study, we investigated whether VG has the potential to covalently bind to macromolecules in cells, a process that could initiate immune-mediated hepatotoxicity. For comparison, M20.7, a major metabolite of VG, and other DPP-4 inhibitors were also evaluated. We found that VG and anagliptin (ANG), which both contain a cyanopyrrolidine moiety, rapidly reacted in non-enzymatic manners on co-incubation with L-cysteine. Both VG and ANG had half-lives of 20–30 min. In contrast, incubation with GSH, rather than L-cysteine, failed to decrease the concentrations of VG or ANG. M20.7, sitagliptin, linagliptin, and alogliptin, having no cyanopyrrolidine moiety, were stable on incubation with L-cysteine or GSH. Structural analysis of the VG– and ANG–cysteine adducts, designated M407 and M487, respectively, revealed that the nitrile moieties of VG and ANG were irreversibly converted to a thiazoline acid. In conclusion, we found that VG and ANG have the potential to covalently bind to a thiol residue of L-cysteine in proteins. Such binding may lead to unpredictable immune responses in humans. L-Cysteine, rather than GSH, would likely be useful to detect the potential for covalent binding that could initiate immune-mediated hepatotoxicity.

1. Introduction

Drug-induced liver injury (DILI) is a major concern in pharmacotherapy. DILI is the most frequent reason for failure during drug development and the withdrawal of drugs from the market. Most cases of DILI are caused by idiosyncratic reactions: adverse events that occur in a small minority of susceptible patients. Because rare events are unlikely to be detected in early-stage clinical trials, hepatotoxic events often lead to severe hepatic failures or mortalities in later clinical trials or postmarketing. The irreversible covalent binding of a drug or its reactive metabolite to cellular macromolecules often initiates immunological responses that may induce immune-mediated DILI. Reactive intermediates may be formed via drug metabolism catalyzed by enzymes such as cytochrome P450 (CYP) or uridine diphosphate glucuronosyltransferase (UGT). Such reactive intermediates then directly bind to proteins. Some drugs themselves, e.g., penicillin, covalently bind to proteins in nonspecific manners. In the resulting adduct, the drug moiety could be a hapten; i.e., it could induce the generation of neoantigens and trigger immune responses [1,2].

To predict the toxicological potential of drug candidates during

Abbreviations: ALG, alogliptin; ANG, anagliptin; CRP, C-reactive protein; CYP, cytochrome P450; DILI, drug-induced liver injury; DLST, drug-induced lymphocyte stimulation test; DPP-4, dipeptidyl peptidase-4; EMA, European Medicines Agency; ESI, electrospray ionization; GST, glutathione *S*-transferase; HLA, human leukocyte antigen; HLC, human liver cytosol; HLM, human liver microsome; HMBC, heteronuclear multiple bond coherence; IS, internal standard; k_{el}, log remaining ratio versus incubation time relationships; k_{pr}, production rate constant; LC–MS/MS, liquid chromatography–tandem mass spectrometry; LG, linagliptin; MHC, major histocompatibility complex; MRM, multiple reaction monitoring; S100, S100 calcium-binding protein; SG, sitagliptin; T2DM, type 2 diabetes; UGT, uridine diphosphate glucuronosyltransferase; VG, vildagliptin

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drug discovery, the covalent binding of compounds to hepatic proteins is generally examined *in vitro* by incubations of radiolabeled drugs with human liver microsomes (HLM) or hepatocytes [3,4]. The nucleophilic sites of proteins, e.g., the thiol residues of cysteine, are targets for electrophilic intermediates or drugs. GSH and its analogs are used as nucleophilic trapping agents. The GSH conjugates of drugs are hydrolyzed to cysteine conjugates and are then acetylated *in vivo* to form mercapturic acid metabolites. These metabolites are considered to be markers of covalent binding [5,6].

Vildagliptin (VG) is a potent, selective, and orally administrable dipeptidyl peptidase-4 (DPP-4) inhibitor [7]. VG is used worldwide for treating type 2 diabetes (T2DM) [8]. A dose of 50 mg VG twice daily is recommended. A meta-analysis of 38 clinical trials involving T2DM patients suggested that VG (50 mg qd or bid) did not result in an increased risk of DILI [9]. Nonetheless, in rare cases, hepatic dysfunction (including hepatitis) has been observed [10], although the details are not publicly accessible. Recently, Kurita et al. [11] reported a case of liver injury caused by VG in Japan. In this patient, the serum transaminase levels were increased 8 months after the first VG administration (50 mg/day), and the drug-induced lymphocyte stimulation test (DLST) was positive, suggesting that immune responses were associated with the liver injury. Anno et al. [12] reported another DLST-positive case of drug fever and acute inflammation resulting from hypercytokinemia in a patient administered VG (100 mg/day). Other than an increase of C-reactive protein (CRP) levels, no symptoms were observed during the first and second administrations of VG (for 2 and 7 days, respectively). However, the day after the third administration was started, the patient had high fever with elevated CRP levels. In nonclinical studies in animals, VG administration did not result in hepatic abnormalities [10,13]. The mechanisms of human-specific DILI and hypersensitivity remain to be elucidated.

After oral administration, VG is rapidly and well absorbed in both healthy volunteers and patients with T2DM [14]. The primary metabolic pathway of VG is hydrolysis of the nitrile moiety to form the carboxylic acid metabolite M20.7 [15], which is also pharmacologically inactive [7]. Human DPP-4 greatly contributes to the hydrolysis of VG [16]. In humans, minor metabolites are reportedly formed via hydrolysis, glucuronidation, and oxidation [15]. However, GSH-conjugated and related metabolites have not been detected in humans or animals. Using radio-labeled VG, Dahal et al. [17] recently reported that VG has the potential to bind covalently to human hepatocytes. However, the extent of covalent binding was lower than that of other electrophilic drugs. The aim of the current study was to clarify how VG, in comparison with a metabolite of VG and other DPP-4 inhibitors, covalently binds to protein in hepatocytes. The results should help clarify the mechanism of VG-induced liver injury and hypersensitivity.

2. Materials and methods

2.1. Chemicals and reagents

VG and sitagliptin (SG) phosphate monohydrate were purchased from LKT Laboratories (St. Paul, MN). ANG was purchased from Shanghai Haoyuan Chemexpress (Shanghai, China). Linagliptin (LG) and alogliptin (ALG) were purchased from Chem Scene (Monmouth Junction, NJ) and Toronto Research Chemicals (Toronto, Canada), respectively. VG carboxylic acid metabolite (M20.7) was from Santa Cruz Biotechnology (Dallas, TX). GSH and L-cysteine were from Nacalai Tesque (Kyoto, Japan). Pooled HLM (Ultrapool™ HLM 150) and human liver cytosol (HLC, Ultrapool™ Human Cytosol 150) were obtained from Corning (Corning, NY). NADPH and NADH were obtained from Wako Pure Chemical Industries (Osaka, Japan). Other chemicals and reagents used in this study were of analytical grade or the highest grade commercially available.

2.2. Stability assay of VG, its metabolite M20.7, and other DPP-4 inhibitors on incubation with nucleophiles

Incubation mixtures consisted of 1 mg/mL human enzyme source (HLM, HLC, or neither), 1 μM substrate (VG, M20.7, ANG, ALG, SG, or LG), 10 mM trapping reagent (L-cysteine, GSH, or neither), 5 mM $MgCl_2$, and 1 mM NADPH and NADH (as CYP cofactors) in a total volume of 300 μL potassium phosphate buffer (100 mM, pH 7.4). All substrates were dissolved in methanol at 100 μM, and the final fraction of methanol in the mixture was 1%. After pre-incubation for 5 min, the reaction was started by the addition of substrate in a shaking water bath at 37 °C. After incubation for 0–120 min, 20-μL aliquots were collected as analytical samples at each time point. To the collected samples, 500 μL of ice-cold acetonitrile containing 1 ng/mL spiperone as an internal standard (IS) was immediately added and the samples were centrifuged at 6130*g* at 4 °C for 10 min. The supernatant was diluted with the same volume of ice-cold 100 mM sodium phosphate buffer (pH 6.0) in an ice bath and subjected to liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis.

The LC–MS/MS system consisted of a Prominence UFLC system (Shimadzu, Kyoto, Japan) and an API4000 triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source (AB SCIEX, Framingham, MA) and a switching valve (Valco Instruments, Houston, TX). Analyte separation was achieved on a reverse-phase HPLC column XBridge C18 (3.5 μm, 2.1 mm ID × 50 mm, Waters, Milford, MA). The mobile phases consisted of 0.5 mM ammonium hydrogen carbonate aqueous solution (A) and methanol (B) for the separation of VG and M20.7, and 1 mM ammonium acetate aqueous solution (A) and acetonitrile (B) for ANG, ALG, SG, and LG. The flow rate was 0.55 mL/min. Linear-gradient elution was performed as follows: 5% B at 0–0.1 min; 5–90% B at 0.1–1.0 min, and 90% B at 1.0–2.0 min. The autosampler tray was kept at 4 °C. The mass spectrometer was operated in ESI positive ion mode using multiple reaction monitoring (MRM) transitions at *m/z* 304.2/154 for VG, 323.2/173 for M20.7, 384.2/160 for ANG, 340.2/89 for ALG, 408.1/235 for SG, 473.2/420 for LG, and 396.0/165 for spiperone. Quantification of each analyte was achieved by comparison of the analyte/IS peak area ratios to those of a calibration curve ranging from 0.01 μM to 2 μM. The half-lives of unchanged drug were calculated from the slope of the linear regression line for the log remaining ratio versus incubation time relationships ($-k_{el}$) using the following equation: half-life = $0.693/k_{el}$.

2.3. M20.7 formation from VG by human liver fractions

To evaluate whether DPP-4 works adequately in the present assay, M20.7 formation from VG by HLM and HLC was determined. Incubation mixtures consisted of 1 mg/mL HLM or HLC, 10 μM VG, 5 mM $MgCl_2$, and 1 mM NADPH and NADH in a total volume of 300 μL potassium phosphate buffer (100 mM, pH 7.4). NADPH and NADH were not required for M20.7 formation but were added to unify the incubation conditions with that of the stability assay. After pre-incubation for 5 min, the reaction was started by the addition of substrate. After incubation for 120 min, 40-μL aliquots of the mixture were collected and mixed with 500 μL of ice-cold acetonitrile containing 1 ng/mL spiperone. After centrifugation, the supernatant was diluted with ice-cold 100 mM sodium phosphate buffer (pH 6.0) and subjected to LC–MS/MS, as described above. The quantification of M20.7, the hydrolyzed metabolite of VG, was achieved by comparison of the analyte/IS peak area ratios to those of a calibration curve.

2.4. Estimation of the structure of cysteine adducts of VG and ANG by LC–MS

VG or ANG (100 μM) was incubated with or without L-cysteine (100 mM) in a potassium phosphate buffer (100 mM, pH 7.4) at 37 °C for 2 h. Aliquots of the reaction mixture were analyzed using an