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Reaction of Proton Pump Inhibitors With Model Peptides Results in Novel Products

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Abstract. The proposed mechanism for proton pump inhibitors (PPIs) is that PPIs are activated at low pH to the sulfenamide form, which reacts with the sulfhydryl group of cysteine(s) at the active site of the proton pump, to produce reducible disulfide-bonded PPI-proton pump conjugates. However, this mechanism cannot explain the observations that some PPI-protein conjugates are irreducible. This study was designed to investigate the chemistry of the irreducible conjugates by mass spectrometry, using three PPIs and 17 cysteine-containing peptides. While some peptides favored the formation of reducible PPI-peptide adduct, the other peptides mainly produced irreducible adducts. Characterization of the irreducible adduct revealed that the irreducible bonding required the participation of both a sulfhydryl group and a nearby primary amino group. High resolution mass spectrometry suggested a molecular structure of the irreducible adduct. These results suggested a reaction mechanism in which the PPI pyridone form reacted with an amino group and a sulfhydryl group to form an irreducible adduct. The irreducible adduct becomes the dominant product over time because of the irreversible nature of the pyridone-mediated reaction. These findings may explain the irreducible inhibition of H/K-ATPase by PPIs and their relatively slow biological turnover in vivo.
[Supplementary materials: available only at http://dx.doi.org/10.1254/jphs.13058FP]

Keywords: proton pump inhibitor (PPI), omeprazole, lansoprazole, pantoprazole, mass spectrometry

Introduction

Lansoprazole, omeprazole, and a number of analogs, called substituted benzimidazoles, comprise a family of structurally related compounds that are widely used as therapeutic agents to inhibit gastric acid secretion by reacting with the proton pump H,K-ATPase. These drugs are generally known as proton pump inhibitors (PPIs), and their major mechanism of action to inhibit the gastric proton pump has been extensively studied (1 – 6). The consensus has been that the acid-activated PPIs react with a few sulfhydryl groups on the apical surface of the acid-secreting cells, forming a disulfide bond that is readily reducible by accessible thiols. The structures of the three PPIs used in the current study are shown in Fig. 1. In neutral form, the benzimidazole is interlocked through a carbon sulfur chain with a pyridine ring. The differences among the various related drugs come from the chemical substituents on the rings, as indicated here for lansoprazole, omeprazole, and pantoprazole. These substituents, in turn, affect solubility, reactivity, and biological longevity of the individual drug.

One of the striking facts about the substituted benzimidazoles is that they are inactive in neutral plasma-like solutions, but under acidic conditions they undergo a significant rearrangement of molecular structure leading to the loss of a proton and a water molecule and an important change in biological reactivity as shown in Fig. 1. Originally this was thought to be a simple reduction (4), but more recent studies suggest the bond linking the benzimidazole to the pyridine ring goes through a more complicated shuffling (7). In any case, the rear-

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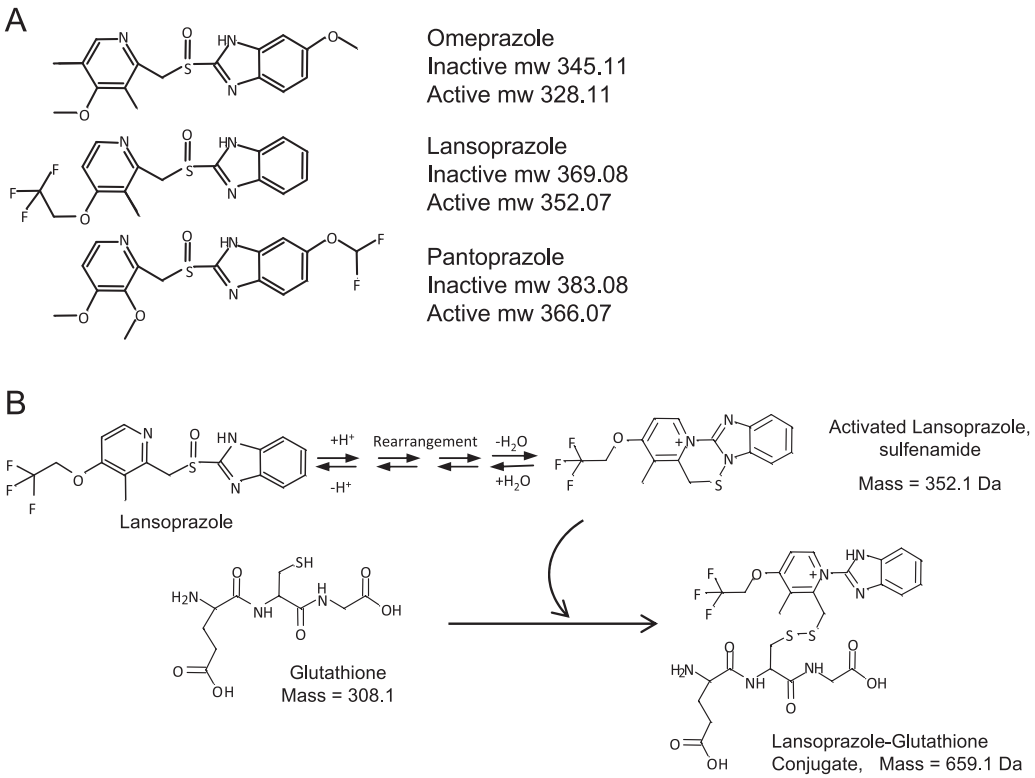


Fig. 1. Commonly accepted reaction mechanism of PPI with cysteine-containing peptide. A) The structures of the neutral (inactive) forms of the PPIs used in this study. The molecular weights of the neutral and acid-activated forms are shown on the right side. B) When exposed to acidic conditions, substituted benzimidazoles (lansoprazole in this example) rapidly converts to the reactive sulfenamide form, which reacts with the cysteine in the peptide glutathione, forming a disulfide bond.

rangement/shuffling results in a drug that is both charged and reactive with exposed cysteine residues.

The conventional understanding of the reaction between the PPIs and the cysteines of the H,K-ATPase has not changed appreciably in the past 20 years (8). As summarized in Fig. 1, it has been proposed that the acid-activated, sulfenamide form of the drug forms disulfide bonds with the sulfhydryl group(s) of two critical cysteine residue(s) of the α-subunit of H,K-ATPase and such a disulfide bond is reversible by reducing agents (8). Although this mechanism has gained wide acceptance, there is some evidence that more complicated reactions between the PPI's and the H,K-ATPase may also occur. For example, Shin and Sachs have found that, unlike omeprazole and lansoprazole, inhibition of H,K-ATPase by pantoprazole was not reversible by even high levels of reducing agents, e.g., 100 mM β-mercaptoethanol (9). This result suggested that there might be some differences among the PPIs in how they reacted with the H,K-ATPase. To explore this possibility we used mass spectrometry to analyze the products of the PPIs (lansoprazole, omeprazole, and pantoprazole) in reactions with 17 cysteine-containing peptides under well-controlled conditions. Peptides were chosen or designed to have a cysteine residue located at different locations, N-terminus, C-terminus, or in the middle; and the peptides were of various lengths. Other than these, the peptide sequences were chosen at random for the purpose of proving a general principle. Our data show that all three of these drugs formed non-reducible and irreversible adducts with peptides through the pyridone form of the drugs. The results have implications on the fate and biological turnover of the PPIs in vivo.

Methods and Materials

Materials

Pure omeprazole, lansoprazole, and pantoprazole were obtained from Sigma (St. Louis, MO, USA). Immobilized tri(2-carboxyethyl) phosphine hydrochloride (TCEP) was from Pierce. Sequencing grade, side-chain protected porcine trypsin was from Promega (Madison, MI, USA). Iodoacetamide was obtained from Sigma. Alpha-cyano-4-hydroxycinnamic acid and 2,5-dihydroxy benzoic acid MALDI-matrix solutions were obtained from Agilent Technologies (Santa Clara, CA, USA). Both stock and

custom peptides were procured from the American Peptide Company (Santa Clara, CA, USA). All other chemicals were of the highest reagent grade available.

Peptide labeling

A 6-μl aliquot of omeprazole, lansoprazole, or pantoprazole (1 μg/μl in ethanol) was added to 3 μl aqueous HCl solution (pH 2.0) containing 2 nanomoles peptide, followed by a 20-min incubation for acid activation. The final pH of this solution was 3.0. The solution turned dark brown after incubation. Then 80 μl of a pH 7.5, 50 mM sodium phosphate buffer was added, and this solution was incubated for 2 h at 37°C. This was done to mimic the situation in vivo when the gastric luminal pH increases upon inhibition of the proton pumps. Slight variations of this procedure were given for individual experiments.

For MALDI analysis, the sodium phosphate buffer was replaced with 80 μl of 6.1 mM ammonium bicarbonate buffer (pH 7.8). Due to a lack of buffering power under these conditions, the pH of the final solution was lowered approximately1 pH unit by the residual HCl from the PPI activation.

Acetylation

Peptides were suspended in 25 mM sodium phosphate, pH 7.1. Acetic anhydride was added in 10-fold excess of the peptide and the reaction mixture incubated for 1 h at 37°C. The acetylated peptides were purified by HPLC. The HPLC peptide fractions were diluted 20% by volume with ammonium bicarbonate, evaporated down, and resuspended in buffer. Immobilized TCEP (20% by volume) was added to reduce oxidized cysteine, and the samples were vortexed for 1 h. Activated drug was added, and the immobilized TCEP was removed by centrifugation. Incubation proceeded for 2 h at 37°C.

Alkylation

Peptide (2 nmoles) was suspended in 25 mM ammonium bicarbonate and incubated with 6 mM DTT for 30 min. The solution was then brought to 55 mM iodoacetamide and incubated at 25°C in the dark for 1 h.

Mass spectrometry

Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry was performed on a Perceptive Biosystems Voyager DESTR instrument in the reflectron mode. Both CHCA (alpha-cyano-4-hydroxycinnamic acid) and DHB (2,5-dihydroxybenzoic acid) were used as matrices. MALDI-TOF was used for initial screening and sequencing of samples with MH^+ values of *m/z* 500 and larger.

Initial measurements of sample masses with molecular weights between 100 – 500 Da were made with electrospray on a Perceptive Biosystems Mariner Biospectrometry Workstation, an electrospray-orthogonal-acceleration-time-of-flight mass spectrometer. This instrument was also used to monitor the transformation of PPIs in solution to the activated form. Samples were infused at 1 μl/min in a 50% acetonitrile 0.1% formic acid solution.

Subsequent screening and time courses were run on a Waters ZQ mass LC/MS system (Waters, Milford, MA, USA) equipped with a Higgins Analytical TARGA C18 3 μm 50 × 2.1 mm Column (P/N: TS-0521-C183). Mobile phases were 0.1% formic acid and 0.1% formic acid in acetonitrile. Linear gradients of 5% – 95% acetonitrile in 10 min and 5% – 47% acetonitrile in 20 min were used. A 1-min wash at 95% acetonitrile followed by 5-min equilibration at 5% acetonitrile was used at the end of each gradient.

Peptide sequencing was conducted on a PE Sciex API QSTAR Pulsar quadrupole-orthogonal-acceleration-time-of-flight hybrid tandem mass spectrometer. LC MS/MS analysis [consisting of an HPLC system coupled in tandem directly to the mass spectrometer, so that as the peptides elute they are infused directly into a mass spectrometer for mass measurement as well as for the collision-induced dissociation (CID) experiment] was also conducted on this instrument with an Agilent 1100 series nano HPLC system.

A Thermo Finnegan Orbitrap XL was used for high resolution mass measurement, higher energy collision dissociation (HCD), and CID sequencing of CDP peptide conjugates. LC/MS^{nth} was conducted by infusion. Other measurements were conducted by LC/MS utilizing a Higgins Analytical TARGA C18 5 μm 250 × 0.150 μm Column (P/N: TC-2515-C185) trimmed to 10 cm.

L isolation and incubation

Purification of the PPI breakdown products, the L (and L') pyridones, was conducted on the LC elements of a Waters ZQ mass LC/MS system. Mobile phases were 0.1% formic acid and 0.1% formic acid in acetonitrile. A linear gradient of 5% to 95% acetonitrile in 20 min was used and the peak at approximately 17.5 min collected. A 1-min wash at 95% acetonitrile followed by 5-min equilibration at 5% acetonitrile was used at the end of each gradient. A Higgins Analytical TARGA C18 3 μm 50 × 2.1 mm Column (P/N: TS-0521-C183) was used. The isolated L pyridones were then mixed with the CDPGYIGSR peptide in an aqueous buffer at pH 5.0, and the mixture was taken to complete dryness in a speed vac. The pellet was resuspended in 80 μl of a pH 7.5, 50 mM sodium phosphate buffer. Incubation proceeded for 2 h at 37°C.

Table 1. Two types of PPI-peptide adducts

A) Peptide forming reducible adducts Peptide sequence	Molecular weight $[M + H]^{+1}$	Mass observed when reacted to Lansoprazole*	Increased mass when reacted to		
			Lansoprazole	Omeprazole	Pantoprazole
ECG	308.1	659.2	351.1	327.1	365.1
RGDC	450.2	801.3	351.1	327.1	365.1
GRGDSPC	691.3	1042.4	351.1	327.1	365.1
DRVYIHPCHLLYYS	1778.9	2130.0	351.1	327.1	365.1
GPKTPEKTANTISKFDC	1836.9	2188.0	351.1	327.1	365.1
ac-GPKTPEKTANTISKFDC	1878.9	2206.0	327.1	327.1	365.1
YRVRFLAKEQVTNDAEDNC	2271.1	2622.2	351.1	327.1	365.1
ac-CDPGYIGSR	1009.4	1360.5	351.1	327.1	nm
ac-CNLSPTTIETAMNSPYSAE	2070.9	2398.0	327.1	327.1	nm

B) Peptide forming irreducible adducts Peptide sequence	Molecular weight $[M + H]^{+1}$	Mass observed when reacted to Lansoprazole	Increased mass when reacted to		
			Lansoprazole	Omeprazole	Pantoprazole
C	122.0	355.1	233.1	277.1	315.1
CDP	334.1	567.2	233.1	277.1	315.1
CLRRASLG	875.5	1108.6	233.1	277.1	315.1
CDPGYIGSR	967.4	1200.5	233.1	277.1	315.1
CNDQIKKM	979.5	1212.6	233.1	277.1	315.1
CQDSETRTFY	1249.5	1482.6	233.1	277.1	315.1
CGYGPKKKRKVGG	1377.8	1610.9	233.1	277.1	nm
CKQLQKDKQVYRATHR	2002.1	2235.2	233.1	277.1	315.1

*Molecular mass determined by MALDI-TOF MS. nm: not measured.

Results

Reaction of substituted benzimidazoles with cysteine-containing peptides: reducible and irreducible adducts

Glutathione is a simple three amino acid peptide that can be used for drug-labeling analysis of a thiol. Table 1A shows the results of the mass spectrum analysis of the PPI-glutathione adduct when glutathione was mixed with the activated lansoprazole. The observed *m/z* corresponds to the mass of glutathione, 307.3 Da plus an additional 351.1 Da, which is the expected mass increment of lansoprazole conjugated to the cysteine in a disulfide bond. Major adducts of *m/z* at 659.2, 635.2, and 673.1 were observed following incubation of glutathione with lansoprazole, omeprazole, and pantoprazole, respectively. The detected masses were consistent with the formation of disulfide bonded adducts of PPIs and glutathione. In all three cases these adducts were readily removable with a 10-fold excess of reducing agent (DTT or TCEP) over labeling agent (data not shown).

We then tested for PPI-labeling of a series of larger cysteine-containing peptides. Expected and unexpected adducts were observed (summarized in Table 1). Table 1A lists peptides whose major products were disulfide-bonded adducts when reacted with a PPI molecule. The mass increases of the peptides were 351.1, 327.1, and 365.1 Da when reacted with lansoprazole, omeprazole, and pantoprazole, respectively. All peptides listed in Table 1A are similar to glutathione in that they formed PPI adducts that were readily removed with the addition of 10 mM DTT, indicating the formation of disulfide-bonded, reducible adduct following the mechanism specified in Fig. 1.

A second group of cysteine-containing peptides produced unexpected adducts as major products when reacted with PPIs (summarized in Table 1B). For example, when activated lansoprazole was reacted with the peptide CDPGYIGSR, only a small amount of the expected disulfide bonded PPI-peptide adduct was observed at *m/z* 1318.5 Da (967.4 + 351.l). Instead, the major adduct was observed at *m/z* of 1200.5 Da. Similar results were obtained for all the other peptides in Table 1B: the final adducts from these peptides were smaller than the predicted value for the conventionally reacted peptides listed in Table 1A, and there was a different characteristic mass for each PPI. The difference between predicted increases in mass and observed increases in mass was 118.0 Da for lansoprazole (i.e., 351.1 – 233.1) and 50.0

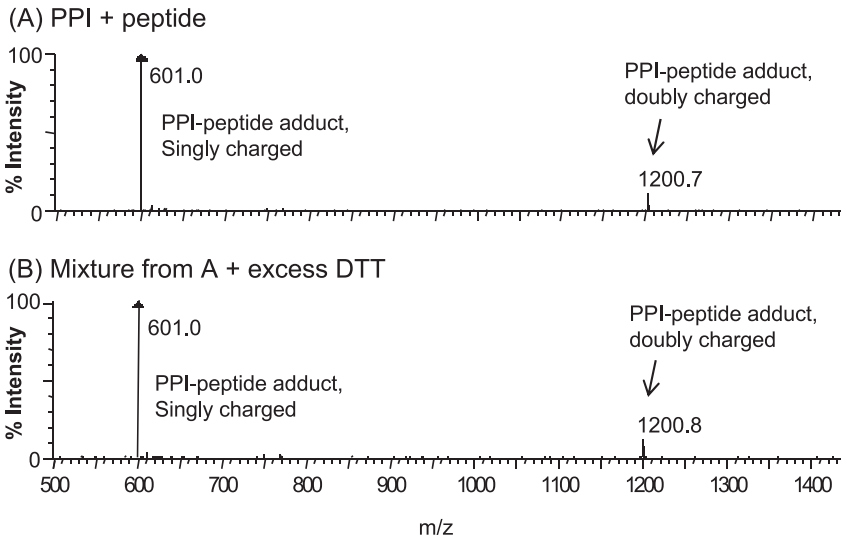


Fig. 2. Formation of irreducible adduct. A) The MALDI spectrum from the product of lansoprazole reacted with the peptide CDPGYIGSR. The irreducible adduct flies as the singly and doubly charged *m/z* at 1200.7 and 601.0, respectively. B) An aliquot of the product from A was incubated with 2M DTT. The same adduct as in A were observed.

Da for omeprazole (327.1 – 277.1) and pantoprazole (365.1 – 315.1). The magnitudes of these differences suggested that this change in molecular weight during reaction with the activated PPIs could be explained by deletion of the fluorethoxy of lansoprazole and the methoxy of the omeprazole and pantoprazole substituents on the pyridine ring of these compounds.

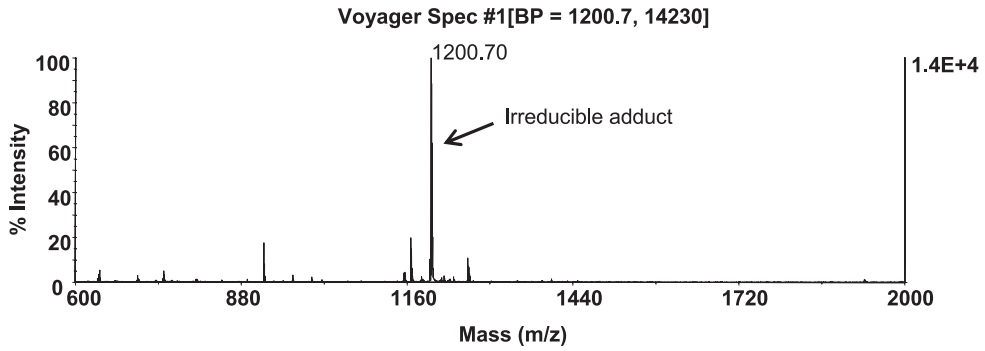
Determination of the structure of the irreducible adduct

A surprising observation with the unexpected adducts was that they were irreducible. MALDI spectra in Fig. 2 showed that the lansoprazole–peptide CDPGYIGSR adduct (*m/z* = 1200.7) was irreducible following treatment with 2 M DTT (5000 × excess over the reactants) for 1 h. Similar irreducibility was observed following treatment with 20 mM TCEP for 1 h. This irreducibility was unlike the adducts formed with glutathione and other peptides listed in Table 1A, which were readily reversed by 10 mM DTT. Furthermore, when acid-activated PPIs were incubated with N-terminal cysteine-containing peptides in the presence of DTT, no adduct at *m/z* = 1200.7 was formed (Fig. 3), demonstrating that the cysteine SH group was required for the formation of the unconventional adducts. Further evidence for the participation of the SH group in the formation of the irreducible adduct was that the adduct could not be carboxymethylated by iodoacetamide, consistent with the absence of the SH group in the irreducible adduct (Supplementary Fig. 1: available in the online version only).

Formation of the irreducible adduct also appears to require a primary amine in the vicinity of the cysteine sulfhydryl group. This was initially suggested by the fact that the N-acetylated cysteine peptide Ac-CNLSPTTIETAMNSPYSAE (Table 1A) formed the conventional disulfide-bonded adduct. We therefore tested the reaction of the irreversible product of peptide CDPGYIGSR with the acetylation reagent acetic anhydride. When the test peptide was reacted with activated lansoprazole, an irreducible adduct was observed, with some unadducted peptide monomer and dimer still remaining (Fig. 4A). After introducing acetic anhydride into the mixture, the monomeric and dimeric forms of the original peptide were readily acetylated, but the PPI-peptide adduct did not react with acetic anhydride (Fig. 4B), suggesting that the N-terminal amino group was no longer present in the irreducible adduct. Another line of evidence supporting the participation of a nearby amino group in the formation of the irreducible adduct is that peptide-acetylation (blocking the amino groups) prevented the formation of irreducible adduct (Supplementary Fig. 2: available in the online version only).

For better insight into the composition and structure of the irreducible adducts, a reaction mixture containing lansoprazole and lansoprazole conjugated to CDP peptide in both the reducible and irreducible forms was examined on a high resolution Orbitrap XL mass spectrometer. The high resolution spectrum in the mass region for each of these components is presented in Fig. 5. For both activated lansoprazole and the CDP adducts, the observed mass and the theoretical mass were in excellent agreement, with a difference of 2.15 – 3.14 parts per million of the theoretical mass. This accuracy allowed us to calculate the elemental composition of the irreducible CDP-lansoprazole to be C26H27N6O7S1, based on the elemental compositions of the starting materials. Similar calculations and results were obtained for the irreducible conjugates of the other PPIs (data not shown).

(A) Formation of the irreducible product with lansoprazole and the CDPGYIGSR peptide



(B) Reaction of lansoprazole with the CDPGYIGSR peptide in the presence of DTT

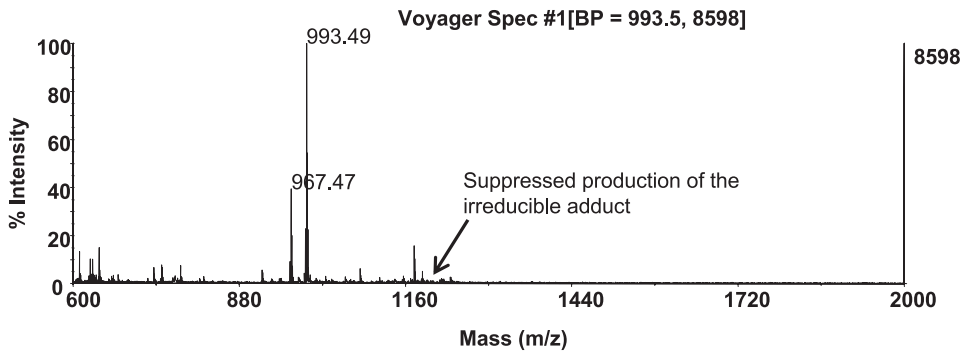
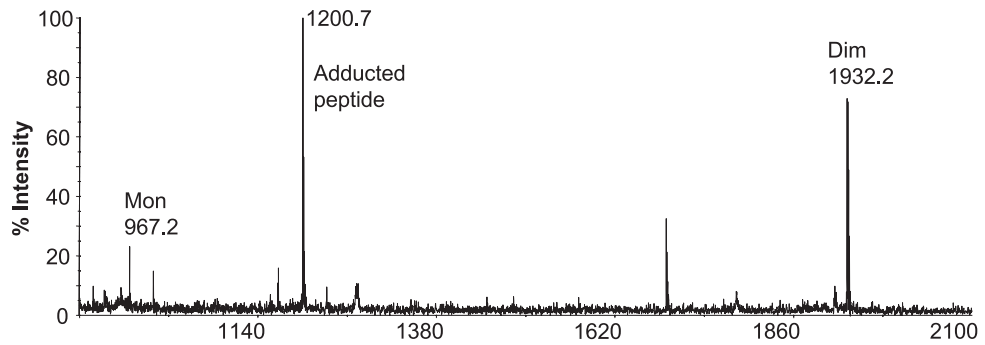


Fig. 3. The sulfhydryl group is required for the production of the irreducible adduct. A) Activated lansoprazole was reacted with the peptide CDPGYIGSR. The MALDI spectrum shows the irreducible adduct at the *m/z* of 1200.7 Da. B) A similar reaction was performed in the presence of DTT at 10-fold excess. The irreducible adduct was not detected.

(A) Prior to acetylation



(B) After acetylation

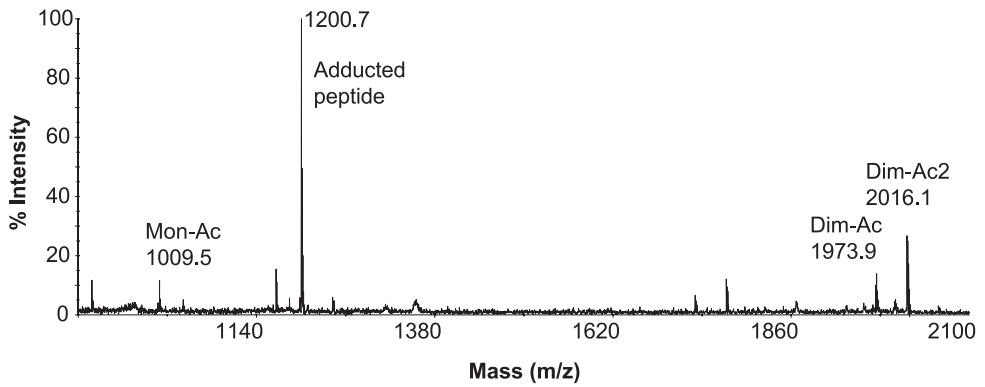


Fig. 4. The irreducible bond with PPI prevents N-terminal acetylation of cysteine-containing peptide. A) Peptide CDPGYIGSR was reacted with activated lansoprazole. Irreducible peptide-lansoprazole adduct was observed with MALDI at a *m/z* of 1200.7. Some unreacted monomeric (Mon) and dimeric (Dim) peptides were also observed at 967.2 and 1932.2, respectively. B) An aliquot of the product from A was treated with acetic anhydride in 10-fold excess over drug. The residual peptides that were not conjugated to lansoprazole were readily acetylated on their N-termini (masses 1009.5 and 1973.9 plus a mass of 2016.1 for the doubly acetylated dimer). The peptide that was conjugated to lansoprazole at mass 1200.7 did not react with the acetylating reagent; the predicted mass of 1242.6 was not found.

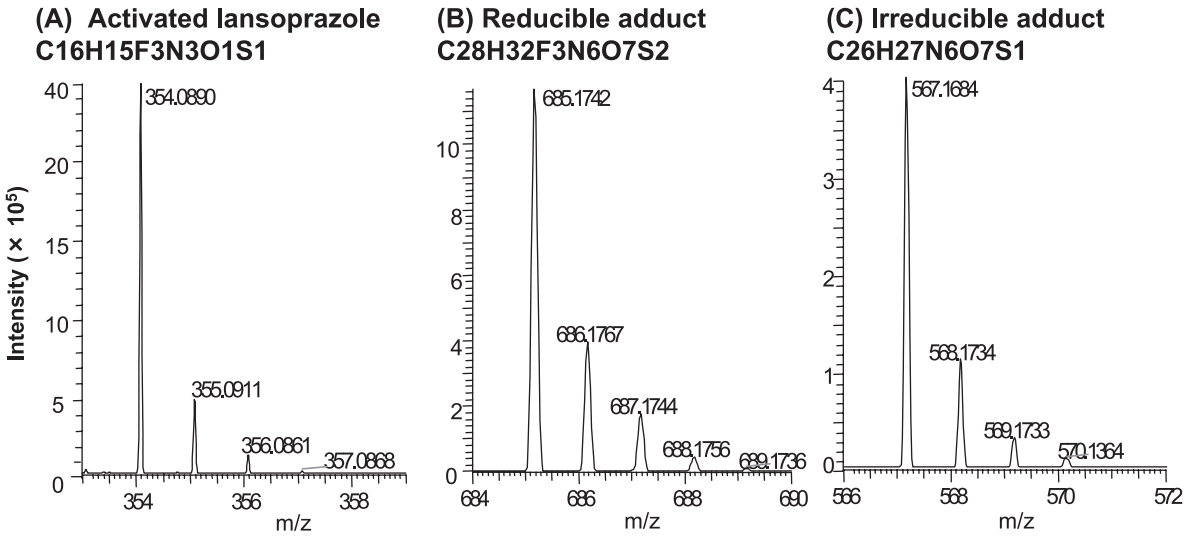


Fig. 5. High resolution Orbitrap mass spectra of lansoprazole and lansoprazole-CDP peptide adducts. A) Acid-activated lansoprazole. B) Reducible lansoprazole-CDP adduct. C) Irreducible lansoprazole-CDP adduct. In each case the observed mass is between 2.15 and 3.85 ppm higher than the theoretical value. Based on starting composition of the reversible product and mass accuracy, a proposed elemental composition is suggested for each compound as indicated.

The mass change from the starting material to the irreducible adduct indicated that the bond involved the number 4 carbon of the pyridine ring. Each of the PPIs we tested had a substitution on that number 4 carbon: $-OCH_2CF_3$ (MW: 99 Da) in the case of lansoprazole and $-OCH_3$ (MW: 31 Da) in the cases of omeprazole and pantoprazole. The mass differences of these leaving groups on number 4 carbon of the pyridine ring exactly explain the differences in the molecular mass of the irreducible adduct formed with different PPIs (Table 1B).

Based on the structures of the rearrangement products of omeprazole as described by Brandstrom et al. (2), we proposed a structure for the irreducible adduct as depicted in Fig. 6, which is formed between the pyridone form of lansoprazole and peptide CDP. This structure is in agreement with the elemental composition being C26H27N6O7S1. To test this model, extensive fragmentation analysis of both CDP-lansoprazole and CDP-omeprazole irreducible adducts were analyzed on an Orbitrap XL mass spectrometer under a variety of CID on HCD conditions. The structures for the fragmentation products were proposed based on the highly accurate mass measurement (Supplementary Table 1 and Supplementary Fig. 3: available in the online version only). These structures and the MS/MS data are consistent with the proposed structural model (Fig. 6).

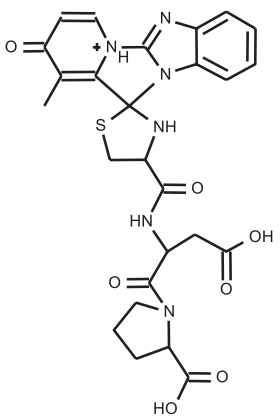


Fig. 6. Chemical structure of the irreducible lansoprazole-CDP adduct suggested by high resolution mass spectrometry analysis. The elemental composition of the adduct is C26H27N6O7S1.

Investigation of the reaction mechanism for the formation of irreducible PPI-peptide adduct

The observation of the L pyridone (2) in the CID spectra of the irreducible PPI-peptide adducts (Supplementary Fig. 3) prompted us to evaluate the possibility that the irreducible adduct is a product of PPI L-form. In several experiments, we observed a molecule of mass 268.1 Da (e.g., Supplementary Fig. 3). This mass was identical to the mass of the hypothetical L analogue for lansoprazole. Being relatively hydrophobic compared to other components of our reaction mixtures, this L pyridone was readily purified for reaction with target peptide and resulted in the production of the irreducible adduct as the major adduct (Fig. 7). Further studies confirmed the existence of the analogue of the L form for other PPIs (data not shown).

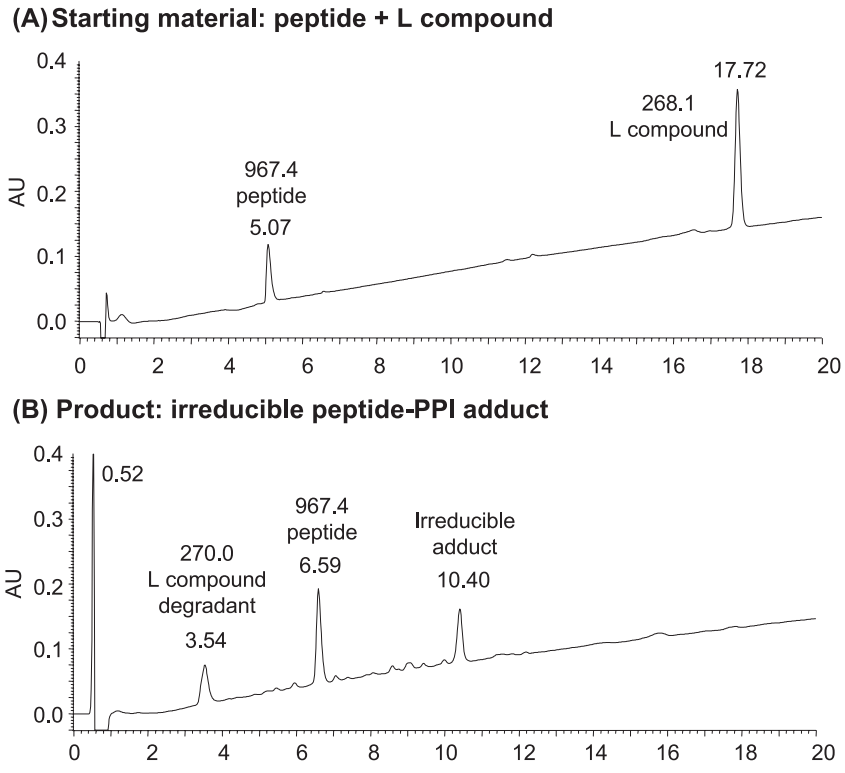


Fig. 7. Formation of the irreducible adduct by reacting the PPI pyridone with peptide CDPGYIGSR. HPLC analysis of the starting material (A): mixture of purified 268.1 Da L pyridone with peptide CDPGYIGSR and the product (B). The integrated UV absorbance (AU) at 214 nm is shown.

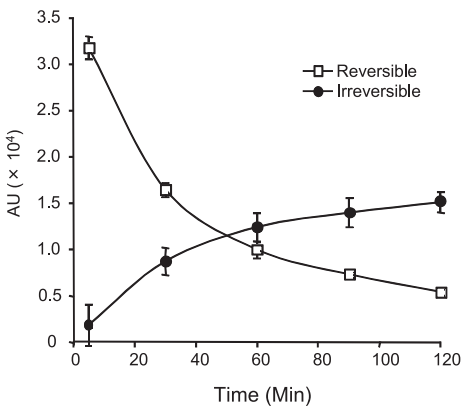


Fig. 8. Time course analysis of the lansoprazole reaction with peptide CDPGYIGSR. Lansoprazole was first activated at pH 3 for 20 min in the presence of target peptide with a drug-to-peptide ratio of 9 to 1. This reaction mixture was then titrated to pH 7.5. Incubation proceeded for 2 h with aliquots examined at various time points by LC/MS as indicated. The integrated UV absorbance (AU) at 214 nm was measured for each product and the results reported for three tests. Error bars: standard deviations.

For further insight into the reaction mechanism of the irreducible adduct formation, time course experiments were performed with all three PPIs and the peptide CDPGYIGSR. Figure 8 shows the result for lansoprazole. Since irreducible adducts were always the major product with CDPGYIGSR in previous experiments, it was surprising that the major product from the 5-min sample was mainly the reducible disulfide-bonded adduct (1318.5 Da). This product diminished rapidly in 30 min and continued to decrease during the ensuing 90 min (total 120 min) of the experiment, with a concomitant increase of the irreducible adduct (1200.5 Da). The irreducible adduct became the major product after 60-min incubation (Fig. 8). Similar results were obtained with omeprazole and pantoprazole (data not shown).

Discussion

Identification and characterization of irreducible PPI-peptide adduct

It is shown here that PPIs can react with cysteine-containing peptides to form irreversible conjugates that are resistant to reduction and different from the disulfide-bonded products that have typically been described by other authors (1, 6, 10 – 13). While the reducible, disulfide-bonded adduct is a product of the sulfenamide form of PPI, the irreducible adduct is likely a product of the pyridone form (L form) of PPI.

Structural studies of the irreducible adduct suggested that a cysteine sulfhydryl group and a vicinal primary amino group on the peptide are needed to form the irreducible adducts presented in this paper. The participation of the sulfhydryl group in the formation of irreducible adduct is suggested by the following: i) No

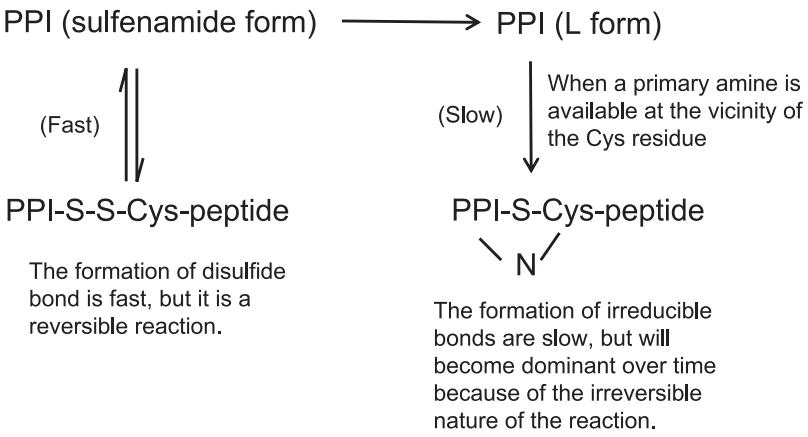


Fig. 9. Proposed mechanism for the production of the irreducible PPI-peptide adduct. The formation of the disulfide bond between the sulfenamide form of PPI and a Cys-containing peptide is fast and thus is usually the dominant reaction. The reaction of the L pyridone of PPI with a Cys-containing peptide is slow and thus is usually a minor reaction. However, when a primary amine is available at the vicinity of the Cys residue, the L pyridone forms an irreducible adduct with the peptide involving both the sulfhydryl group and the amino group. Because of the irreversible nature of this reaction, the balance of the reactions is shifted toward the L pyridone reaction and the irreducible adduct.

adduct was formed when the peptides were kept in a highly reducing environment before and during the reaction with PPIs. ii) The irreversible adducts were not susceptible to alkylation, consistent with absence of the disulfide bond. Evidence for the participation of a vicinal amino group include: i) Amino group is absent from the irreducible adduct, as demonstrated by the fact that the adduct cannot be acetylated. ii) Acetylation of the amino group in the peptide prevented the formation of the irreducible adduct, leaving the reducible adduct in place.

Determination of the elemental composition of the irreducible adduct by high resolution mass spectrometry suggested the structural model shown in Fig. 6. This model suggested that the irreducible adduct was the product of the pyridone form (L form) of PPIs, which was validated by reacting the purified pyridone with an N-terminal cysteine–containing peptide.

The N-terminal Cys-containing peptide can also react with the sulfenamide form of PPIs to produce reducible adducts. Time course experiments indicated that the reversible adduct was the dominant product in the early phase of the reaction. We hypothesize that the reaction involving the sulfenamide is faster than the reaction mediated by the pyridone (L form). However, since the sulfenamide reaction is reversible while the pyridone reaction is irreversible, the product of the pyridone reaction (irreducible adduct) eventually becomes the dominant product (Fig. 9).

In vivo implications

While substituted benzimidazoles bind and inhibit H,K-ATPase both in vivo and in vitro, mild reductive conditions can remove most of them, and the reaction shown in Fig. 1 can account for i) the mechanism of action and ii) the structure of the final product. However, it has been suggested, especially for pantoprazole, that there may be additional or alternative reactions with H,K-ATPase that are not reversed by mild reducing conditions (14 – 16). We have described a novel mechanism of reaction between PPIs and certain peptides that results in a product that cannot be reduced. The reaction is mediated by the pyridone form of PPI, which reacts with the cysteine sulfhydryl group of the peptide and a vicinal free primary amine, generating a thiazolidine ring. It is possible that pantoprazole interaction with H,K-ATPase is dominated by the pyridone-mediated reaction, thus explaining its insensitivity to reducing agents as reported by Shin et al. (16).

However, there are several caveats to be addressed before we can accept this possibility. First, the irreducible reaction we saw with appropriate peptides occurred for all three PPIs examined. In contrast, the studies with H,K-ATPase were specific for pantoprazole (16). This difference could be explained by the slight differences in structure among the PPIs leading to variations in accessibility to the appropriate cysteine on the H,K-ATPase. For example, it has been shown that the different PPIs display both similarities and differences with respect to the reactive cysteines on H,K-ATPase (15). Although omeprazole, lansoprazole, and pantoprazole have only slightly different structures, pantoprazole reportedly reacts exclusively with Cys^{813}, whereas all three react commonly with Cys^{822} (15). A second caveat we must address is whether the H,K-ATPase has a suitable free vicinal amine available for reaction to produce the irreducible adduct. The Cys residues in the H,K-ATPase that have been implicated in binding with pantoprazole are Cys^{813} and Cys^{822} (15). Analysis of the primary sequence of the α-subunit indicates the lack of a suitable primary amine group in close proximity to these suspected Cys residues. Although tertiary folding of the enzyme may provide a suitable group for reaction, without a definitive structural model of the enzyme this idea remains speculative. Another potential source of a suitable amine group for interaction with the PPI is the β-subunit of the H,K-ATPase. The β-subunit is known

to form very close and coordinated interaction with the α-subunit, particularly on the extracytoplasmic side where the Cys/PPI reaction is likely to occur. Studies with a structurally sensitive monoclonal antibody suggest that β-subunit amino acid residues 226 – 236 form a close association with the α-subunit, possibly near the 5th and 6th trans-membrane domains (17). There are two Lys residues at positions 235 and 236. The 5th and 6th trans-membrane domains contain Cys^{813} and Cys^{822}, which are proposed to react with PPIs in vivo. Thus, the in vivo reaction of pantoprazole may involve binding to cysteine residues (e.g., 813) of the α-subunit and an amine from the β-subunit (Lys 235 or 236). Finally, it is possible that an amino group of a phospholipid molecule (e.g., phosphatidyl ethanolamine or phosphatidyl serine) within the lipid protein interface may provide the necessary site of reaction.

We recognize that in vivo evidence is needed to prove the proposed reaction mechanism for the irreducible adduct. To this end, we had attempted to isolate the peptide-PPI adduct from stomach tissue after in vivo labeling of the proton pump with PPIs. Unfortunately, we did not obtain any peptide-PPI adduct. The major obstacle was that no appropriate digestion site exists for enzymes other than chymotrypsin in analyzing the region around Cys^{813} and Cys^{822} of the proton pump α-subunit. Chymotrypsin is very unspecific. In addition, the potential targets are two peptides of possible overlapping sequences (Cys^{813} and Cys^{822} are only 8 amino acids apart). The permutations of potential fragments are exponentially increased by these factors, contributing to the dilute-out of the peptide-PPI adduct signal.

In summary, we demonstrated the production of an irreducible PPI-peptide adduct as the dominant product of PPI reaction with cysteine-containing peptides with a primary amine at the vicinity of the sulfhydryl group. The irreducible bond formation involved the pyridone form of PPI, a sulfhydryl group and a vicinal amino group. We postulate that this novel reaction may explain the irreducible inhibition of PPI in vivo.

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