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RESEARCH ARTICLE

Preformulation stability study of the EGFR inhibitor HKI-272 (Neratinib) and mechanism of degradation

Qinghong Lu and Mannching Sherry Ku

Wyeth Research, Pharmaceutical Development, Pearl River, New York, USA

Abstract

The stability in solution of HKI-272 (Neratinib) was studied as a function of pH. The drug is most stable from pH 3 to 4, and degradation rate increases rapidly around pH 6 and appears to approach a maximum asymptotic limit in the range of pH 812. Pseudo first-order reaction kinetics was observed at all pH values. The structure of the major degradation product indicates that it is formed by a cascade of reactions within the dimethylamino crotonamide group of HKI-272. It is assumed that the rate-determining step is the initial isomerization from allyl amine to enamine functionality, followed by hydrolysis and subsequent cyclization to a stable lactam. The maximum change in degradation rate as a function of pH occurs at about pH 6, which corresponds closely to the theoretical pKa value of the dimethylamino group of HKI-272 when accounting for solvent/temperature effects. The observed relationship between pH and degradation rate is discussed, and a self-catalyzed mechanism for the allylamine-enamine isomerization reaction is proposed. The relevance of these findings to other allylamine drugs is discussed in terms of the relative stability of the allylic anion intermediate through which, the isomerization occurs.

Keywords: Degradation, kinetics, crotonamide, allylic amine, enamine, isomerization, intramolecular catalysis, intramolecular cyclization

Introduction

HKI-272 (Neratinib) is an irreversible inhibitor of epidermal growth factor receptor (EGFR, also known as ErbB-1) and human epidermal growth factor receptor-2 (HER-2, also known as ErbB-2). It has shown promising results in clinical trials for the treatment of non-small cell lung (NSCLC) and breast cancers. An important structural feature of the HKI-272 molecular structure is the dimethylamino crotonamide group, which is critical to its biological action and aqueous solubility[1].

In early development of HKI-272, a major degradant was observed (WAY-188954, Figure 1). The structure of the degradant suggested that, the mechanism of its formation involved a series of chemical transformations, the first and presumably rate-determining step being the isomerization from allylamine to enamine within the crotonamide chain of HKI-272. Thus, the allylamine functionality appeared to be a key factor in the degradation pathway. Other drugs possessing the allylamine functionality include analgesics such as pentazocine, levallorphan, nalorphine, and SNC-80[2,3], antimycotics naftifine and terbinafine[4], and calcium entry blocker flunarizine[5]. Thus, the allylamine group is not unique to HKI-272. However, to the best of our knowledge, the stability of other allylamine drugs under basic pH conditions has not been reported. The fact that, such an isomerization reaction occurred in HKI-272 under conditions which were only slightly alkaline, was somewhat unexpected, especially when considering the typical requirement of either a strong base or a transition metal catalyst to afford such isomerizations in allylic amines[6]. As part of a preformulation study of HKI-272, measurements were made of the degradation rate as a function of pH. Based on these findings, a mechanism of HKI-272 degradation is proposed, and its applicability to other allylamine drugs is discussed.

Address for Correspondence: Dr. Qinghong Lu, PhD, Wyeth Research, Pharmaceutical development. E-mail: Qinghong_lu@yahoo.com

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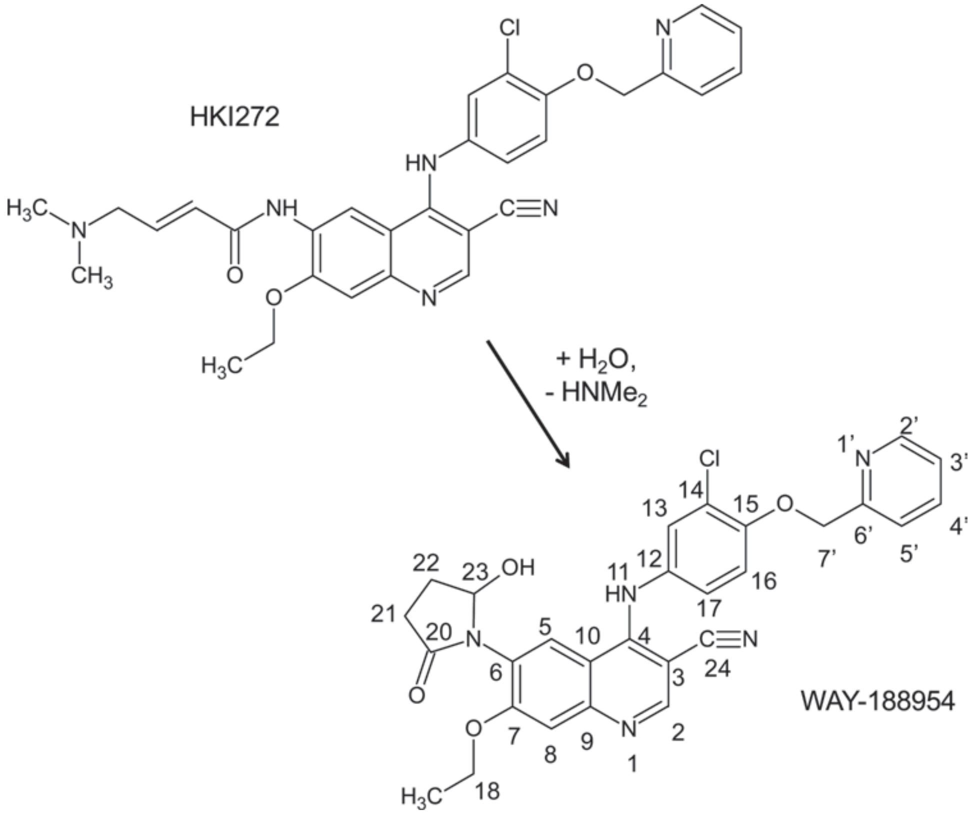

Figure 1. Structure of HKI-272 and degradation product WAY-188954, atoms numbered according to Table 1.

Methods

Degradation product identification

A Waters ZQ single quadrupole mass spectrometer coupled with Waters Alliance (model 2995) HPLC system was used for degradant identification. The HPLC system was also equipped with a photodiode array UV detector (model 2996) for quantitative analysis. Column was YMC-Pack ProC18 (3.5 u, 4.6 × 150 mm) at 30 degrees. Flow rate was 1 mL/min and a gradient was employed from zero to 80% MeCN over 15 min with constant 20 mM ammonium acetate. Retention times were 11.3 min (HKI-272) and 12.2 min (WAY-188954).

NMR spectra were acquired on a Bruker DRX-500 NMR spectrometer equipped with a TXI probe.

pH-stability studies of HKI-272

The pH-stability profile was generated with HKI-272 dissolved at 0.1 mg/mL, in 50 mM phosphate buffers with 50% MeCN (v/v) added to solubilize the API, due to the extremely low solubility of the free base form in aqueous solution[7]. Buffers were first prepared in water by mixing stock solutions of phosphoric acid and mono, di, or tribasic sodium phosphate buffers as needed to obtain target pH within +/− 0.1 pH unit. The resulting aqueous buffers were then combined with MeCN in 1/1 (v/v) ratio. Due to the presence of 50% MeCN in the medium, the original ${}^{W}_{W}pH$ values of the buffers before MeCN was added, were converted to the ${}^{S}_{S}pH$ scale by applying a +1 pH unit correction, based on a standard value of 0.2 pKa units per 10% MeCN typical of weak acids used as buffers[8]. The sample solutions were filled and sealed in glass ampoules which were then placed in a thermostatically controlled oven. Samples were pulled and analyzed at predetermined timepoints by HPLC.

Results

Identification of WAY-188954

The most significant degradation product in all samples had a molecular mass of 529 as determined by LC/MS, and was designated as WAY-188954. In order to be certain of the molecular structure, WAY-188954 was isolated and purified for identification and characterization by NMR spectroscopy. The position of the hydroxyl group in the pyrrolidone ring of WAY-188954 was fairly obvious based on chemical shifts of the two methylene carbons in the ring, having values too low for a carbon adjacent to a nitrogen atom (Figure 1 and Table 1). For further confirmation, however, additional 2D NMR techniques (TOCSY, HSQC, and HMBC) were also used to unambiguously identify the cyclic structure of WAY-188954, which is assumed to be a racemic mixture of the two possible enantiomers.

Kinetics of degradation and calculation of apparent pKa value from rate vs. pH data

Degradation of HKI-272 followed pseudo first-order kinetics for all the pH's and temperatures studied, as shown in Figure 2 in which kinetics data (obtained at

Table 1. The ^{1}H and ^{13}C resonance assignment for WAY-188954 in DMSO-*d*6.

Position	Group	Carbon shift[a]	Proton shift[b]
2	CH	153.67	8.51
3	C	86.04	—
4	C	150.83	—
5	CH	124.57	8.30
6	C	126.53	—
7	C	157.27	—
8	CH	109.58	7.41
9	C	149.82	—
10	C	112.59	—
11	NH	—	9.75
12	C	133.16	—
13	CH	126.82	7.44 (doublet, J = 1.9 Hz)
14	C	121.46	—
15	C	151.62	—
16	CH	114.19	7.27 (doublet, J = 8.7 Hz)
17	CH	125.12	7.24 (dd, J = .7, 1.9 Hz)
18	CH_2	64.28	4.22 (quartet, J = 6.9 Hz)
19	CH_3	14.30	1.39 (triplet, J = 6.9 Hz)
20	C	173.54	—
21	CH_2	28.95	2.59, 2.37
22	CH_2	28.48	2.43, 1.90
23	CH	83.36	5.56
23-OH	OH	—	6.31 (doublet, J = 6.1 Hz)
24	C	117.13	—
2′	CH	149.13	8.60 (doublet, J = 4.7 Hz)
3′	CH	123.07	7.37 (dd, J = 7.7, 4.7 Hz)
4′	CH	137.08	7.87 (triplet, J = 7.7 Hz)
5′	CH	121.46	7.59 (doublet, J = 7.7 Hz)
6′	C	156.12	—
7′	CH_2	71.19	5.30

[a] Shifts relative to DMSO-*d*6 ($\delta^{13}C = 39.5$).
[b] Shifts relative to TMS ($\delta^{1}H = 0.0$).

363 K) were plotted. Since WAY-188954 was the major degradation product in all cases, the initial rate of formation of WAY-188954 mirrored the HKI-272 degradation. The initial rate of WAY-188954 formation as a function of pH changed substantially in the vicinity of ${}_{s}^{s}pH$ 6, appearing to approach asymptotic limits at lower and higher pH (Figure 3). This sigmoidal behavior suggested that, the rate was related to the protonation of the basic dimethylamino group within the HKI-272 structure. In such a case where the free form of an organic base (B) is reactive, while the protonated form (HB^+) is unreactive, the degree of dissociation of the basic group will correlate with the observed reaction rate[9], making it possible to extract an apparent pKa value of the basic group based on the rate versus pH relationship. The expected rate of reaction (r) for a fixed concentration of API is given by Equation 1:

$$r = r_A \alpha$$

where α is the fraction of free amine relative to total of both free and protonated forms $\{B/(B+HB^+)\}$, and r_A is the reaction rate for the reactive species B alone. The value of α can be calculated as a function of $[H^+]$ and the dissociation constant K_D of the protonated amino group HB^+ according to Equation 2

$$\alpha = 1/\{1+([H^+]/K_D HB^+)\}$$

The experimental data for initial rate (r) vs. pH were used to find r_A and apparent pKa values as shown in Table 2, using Equation 1 to calculate r values at each experimental pH. The parameters r_A and pKa were adjusted to derive the best fit between calculated and experimental r values (method of least squares). The experimental data are shown in Figure 3 along with the theoretical function

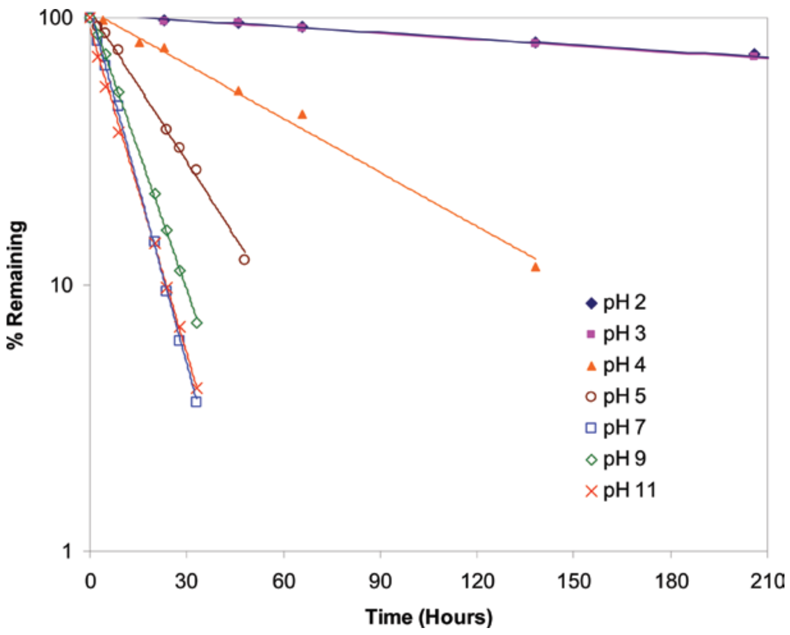

Figure 2. Pseudo first-order kinetics of HKI-272 solutions (obtained at 363 K).

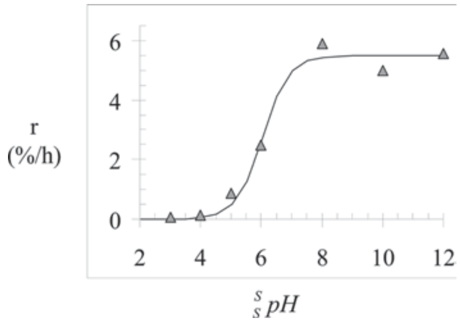

Figure 3. Rate of WAY-188954 formation as function of ${}_{s}^{s}pH$. Solid line shows theoretical function using fitted parameters for ${}_{s}^{s}pH$ and r_A, triangles show experimental data points (Table 2).

Table 2. Data used for finding apparent pKa and r_A values by fitting experimentally obtained *r* values to calculated *r* values.

${}_{s}^{s}pH$	α B/(B+HB$^+$)	*r* (calc.)	*r* (exp.)
3	0.000911	0.0050	0.07
4	0.00904	0.0496	0.12
5	0.0836	0.459	0.86
6	0.477	2.62	2.47
8	0.989	5.43	5.90
10	1	5.49	4.98
12	1	5.49	5.57

Values in column 3 based on apparent pKa of 5.98 and r_A of 5.49 giving best fit between calculated experimental and observed *r* values (columns 4 and 5).

based on the fitted ${}_{s}^{s}pKa$ value of 6.04 and r_A value of 5.49 (%/h).

For comparison, a theoretical ${}_{s}^{s}pKa$ value for the dimethylamino group was calculated with a commercially available computer program (pKalc software, CompuDrug International, Sedona, AZ). The theoretical pKa value (7.65) obtained directly from the software program was substantially higher than the experimentally derived apparent ${}_{s}^{s}pKa$ value, but the theoretical pKa value is relevant for pure water at room temperature conditions, so conversion to the actual experimental conditions (50% MeCN, 363K) is required for valid comparison. It was assumed that MeCN would have a pKa lowering effect of 0.33 units for the dimethylammonium group, based on the reported behavior of a model trialkyl amine compound (triethylamine) in 50% MeCN[10]. It was also assumed that the elevated temperature would cause an additional 1.34 pKa unit reduction, based on the relationship $\Delta pKa/\Delta T = (pKa-0.9)/T$, which gives an estimate of reduction in pKa value of monocationic amine bases[11] due to increased temperature (using average of 298 K and 363 K as the value for T). With the application of both correction factors, we obtain a theoretical temperature-corrected ${}_{s}^{s}pKa$ value of 5.98, in excellent agreement with the apparent ${}_{s}^{s}pKa$ value of 6.04 obtained experimentally from the kinetics data.

Discussion

A mechanism for the HKI-272 degradation is proposed in Figure 4, which shows only the dimethylamino crotonamide part of HKI-272 involved in the degradation. It is assumed that the mechanism involves two unstable intermediates B and C which were not observed directly by HPLC, but their existence is implicated by the position of the hydroxyl group within the observed lactam degradation product D. The first and presumably rate-determining step is allylamine-enamine isomerization from the allylic amine (A) to the enamine (B). It is known that enamines are more thermodynamically stable than the allylic amine counterparts. This is true even in cases where the double bond is conjugated with a carbonyl or aromatic group[12]. Thus, there is a clear driving force for isomerization of crotonamide (A) to enamine (B). Hydrolysis of enamine (B) can be expected to follow[13] in the case of HKI-272 producing dimethylamine and aldehyde (C). Since aldehyde (C) contains the aldehyde group in the 4th position of the amide chain, it is activated towards intramolecular cyclization to a 5-membered lactam product. Intramolecular cyclizations producing 5- or 6-membered rings are known to occur readily due to the favorable ring size[14]. Examples of similar cyclizations include rationally designed amide-carbamate prodrugs[15] which cyclize readily under neutral pH conditions, or numerous cases of unisolated intermediates in N-acylinium ion mediated reactions[16].

Perhaps, the most unexpected aspect of the HKI-272 degradation is that the allylamine-enamine isomerization (A to B, Figure 4) occurs at a slightly alkaline pH, which is counterintuitive considering previous studies of isomerization of allylic amines to enamines. Prior to the development of modern transition metal catalysts, isomerization reactions of allylic amines to enamines were typically catalyzed by alkoxide bases in non-protic solvents[6]. This requirement for a strong base catalyst to generate the allylic anion intermediate reflects the low acidity of the allylic protons. This behavior is evident in the reported reaction half-life of about 2 days for isomerization of allyl dimethylamine at room temperature, even in the presence of 10 mol% alkoxide base in DMSO solvent[17]. While the rate of HKI-272 degradation was also slow (half-life of about 0.3, 1.5, and 4 days at temperatures of 363, 343, and 329 K, respectively, in the ${}_{s}^{s}pH$ range of 812), this substantial reactivity is unexpected considering that no strong base catalyst was present. The most likely cause for the relative ease of isomerization is the presence of the carbonyl functionality in the dimethylamino crotonamide group of HKI-272, in which there is conjugation between a C=C and C=O bond. This conjugated system can be expected to have an activating effect on the ability to generate an allylic anion. For HKI-272, the allylic anion intermediate could be regarded as an extended enolate anion as shown in Figure 5, where the negative charge can be delocalized onto the carbonyl oxygen atom. The combination of double bond conjugation and enolate character will undoubtedly have the effect of making the allylic protons more acidic and stabilizing the allylic anion through charge delocalization[18]. Thus, the carbonyl functionality of HKI-272 likely plays an important role in the observed tendency for the isomerization to occur without any strong base present.

Another important observation during the present study, the substantial pH range in which degradation rate is essentially constant, suggests the possibility that the isomerization step is self-catalyzed by the basic amino group of HKI-272, through either an intermolecular or intramolecular process. The very low concentration of HKI-272 (~0.2 mM) suggests the latter is more likely, but since only one concentration of HKI-272 was used during this study, the former cannot be ruled out. The observed feature of constant rate across a significant pH range is typical of other drugs (such as aspirin) which are capable to decompose via intramolecular catalysis mechanisms[19]. For HKI-272, it is particularly useful to consider an analogy to the hydrolysis of 2-amino benzoate esters, which is a well studied reaction[20]. Like the decomposition of HKI-272, 2-amino benzoate esters exhibit a pH range of constant degradation rate, and a decline in rate corresponding to the degree of amino group protonation. For the 2-aminobenzoate ester hydrolysis, it is proposed that the nucleophilicity of the oxygen atom of a water molecule is increased, due to interaction of a proton from the same water molecule with the basic nitrogen group. For HKI-272, the basicity of an oxygen atom of a water molecule may be increased by interaction of a hydrogen atom of the same water molecule with the basic amino group of HKI-272, promoting the removal of an allylic proton and resulting in formation of the allylic anion. Since this process does not require an external base catalyst and depends only on water, it explains the approximately constant HKI-272 degradation rate in the $_{S}^{S}pH$ range of 8 to 12 (Figure 3), as opposed to the expected four orders of magnitude rate change if hydroxide ion was acting as catalyst. Also, for a self-catalyzed mechanism, the protonation of the amino group would render it catalytically inactive, which is consistent with the observed correlation between degradation rate as function of pH and the pKa of the amino group (Figure 3).

Lacking any studies of other allylamine drugs under basic conditions, it is difficult to generalize the findings of the current study. In principle, other allylamine drugs could also isomerize and hydrolyze into separate amine and aldehyde fragments, analogous to steps A through C (Figure 4). However, with regard to the ease of allylamine-enamine isomerization, HKI-272 is likely an unusually reactive case due to the dimethylamino crotonamide functionality, which enhances the stability of the allylic anion intermediate through the participation of the amide carbonyl group in delocalization of the negative charge. The allylamine olefinic group of other allylamine drugs is either non-conjugated (such as pentazocine or flunarizine), or conjugated with phenyl or alkynyl groups (such as naftifine or terbinafine),

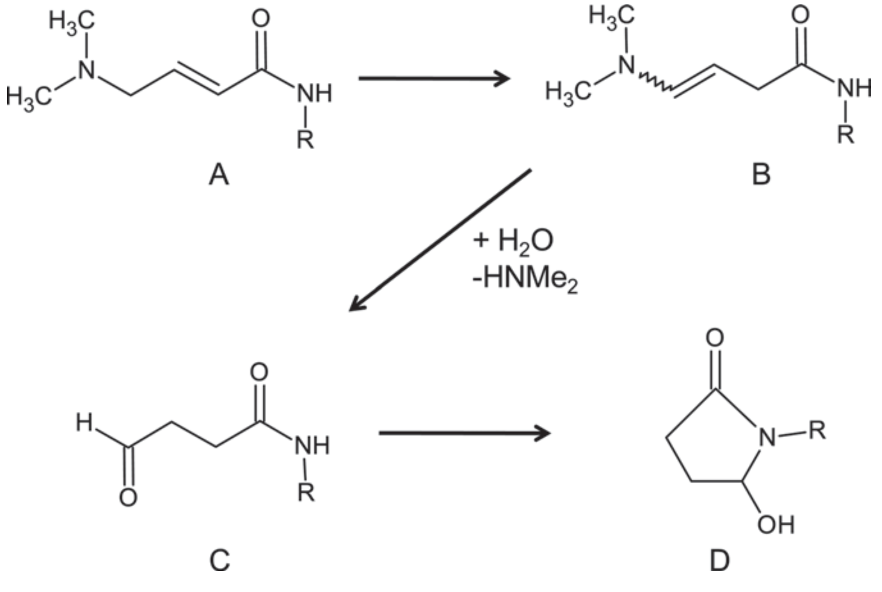

Figure 4. Proposed mechanism of degradation of dimethylamino crotonamide group of HKI-272.

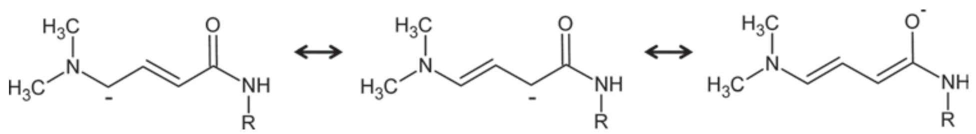

Figure 5. Dimethylamino crotonamide group of HKI-272 showing resonance stabilized anionic intermediate.

which will not produce the same stabilizing effect on the allylic anion intermediate compared to the carbonyl group of HKI-272. Hence, we would expect that other allylamine drugs would be less prone to the allylamine-enamine isomerization. Interestingly, the expected secondary amine degradant resulting from allylamine-enamine isomerization/hydrolysis has been observed for pentazocine[21] and flunarizine[5] in metabolism studies; however, it is important to note that the formation of the amines may have been catalyzed by enzymes present in the biological matrices used for these studies, and thus their appearance does not necessarily implicate the isomerization-hydrolysis pathway. Although degradation in acid has been studied for the same two allylamine drugs pentazocine[22] and flunarizine[23], to our knowledge, no stability studies under basic conditions have been reported. The lack of any previous studies of allylamine drugs under basic conditions may be due to the tendency of such compounds to become much less water soluble at alkaline pH, a problem which could only be overcome in the present study by using an organic co-solvent.

Conclusions

The degradation of HKI-272 was studied in 50% MeCN across the ${}_{S}^{S}pH$ range 3 to 12. In all cases, the main degradation product was WAY-188954. The position of the hydroxyl group in the resulting pyrrolidone ring of the degradant suggests that the degradation mechanism involves the isomerization from allylic amine to enamine and subsequent hydrolysis to an aldehyde intermediate, which undergoes cyclization to the observed lactam. The degradation rate as a function of pH was used to calculate an apparent pKa value, which showed good agreement with a theoretical pKa value for the dimethylamino group of HKI-272. The rate-determining step is assumed to be the allylamine-enamine isomerization step which occurs by an allylic anion intermediate. Based on the essentially constant degradation rate in the ${}_{S}^{S}pH$ range of 8 to 12, and the close correlation between degradation rate and the degree of protonation of the dimethylamino group, a self-catalyzed mechanism is proposed for generation of the allylic anion intermediate, in which water may participate in the net transfer of an allylic proton to the basic amino nitrogen, thereby forming the allylic anion. The presence of conjugation between the amide carbonyl group and olefinic double bond is likely an important factor in stabilizing the allylic anion intermediate of HKI-272, through delocalization of negative charge on the amide oxygen atom, having the effect of making the allylic protons more acidic compared to a non-conjugated allylic amine. This is likely an important contributing factor in allowing the isomerization of HKI-272 to occur without the need for any strong base.

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Declaration of interest

The authors report no conflicts of interest.

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