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Synthesis and structure–activity relationship of α-keto amides as enterovirus 71 3C protease inhibitors

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ABSTRACT

α-Keto amide derivatives as enterovirus 71 (EV71) 3C protease ($3C^{pro}$) inhibitors have been synthesized and assayed for their biochemical and antiviral activities. structure–activity relationship (SAR) study indicated that small moieties were primarily tolerated at P1′ and the introduction of *para*-fluoro benzyl at P2 notably improved the potency of inhibitor. Inhibitors **8v**, **8w** and **8x** exhibited satisfactory activity (IC_{50} = 1.32 ± 0.26 μM, 1.88 ± 0.35 μM and 1.52 ± 0.31 μM, respectively) and favorable CC_{50} values (CC_{50} > 100 μM). α-Keto amide may represent a good choice as a warhead for EV71 $3C^{pro}$ inhibitor.

The human enterovirus 71 (EV71) is one of the primary pathogens of hand, foot and mouth disease (HFMD), which typically infects children under 6 years old.[1] HFMD has caused the highest incidence and death rate among category C infectious diseases in China since 2009.[2] According to the data from Chinese Center for Disease Control and Prevention (CDC), more than 2,778,000 cases of EV71 infections, with 501 deaths, were reported in China in 2014, an increase of almost 50% compared to 2013.[3] Currently, an inactivated EV71 vaccine[4] was approved by China Food and Drug Administration (CFDA) in December 2015. However, no effective antiviral drug is available for treatment or prevention of EV71 infection.[5]

EV71 is a single-stranded, positive-sense RNA of ~7500 nt virus, belongs to the genus enterovirus in the family of Picornaviridae.[6] The viral RNA encodes a polyprotein precursor which is cleaved into four structural proteins (VP1–VP4) to form viral capsid and seven nonstructural proteins (2A–3D) for virus replication.[6a,7] Except for the cleavage of VP1/2A and 3C/3D by 2A protease, 3C protease ($3C^{pro}$) is absolutely required for EV71 polyprotein processing.[8] Meanwhile, $3C^{pro}$ was reported to interfere polyadenylation of host cellular RNA by digesting CstF-64, a critical host factor for 3′-end pre-mRNA processing, suggesting a mechanism by which picornaviruses utilized $3C^{pro}$ to impair host cell function.[9] The pivotal role of $3C^{pro}$ in EV71 infection[10] makes it an attractive target for anti-EV71 drug discovery.

A literature survey of EV71 $3C^{pro}$ inhibitors represented several substrate based reversible or irreversible protease inhibitors (Fig. 1).[2] Rupintrivir (**1**), which failed during Phase II clinical trial as human rhinovirus $3C^{pro}$ inhibitor,[11] was found to possess potently inhibitory activity against EV71 $3C^{pro}$ (IC_{50} = 2.3 ± 0.5 μM).[12] Starting from rupintrivir, derivative **2** with

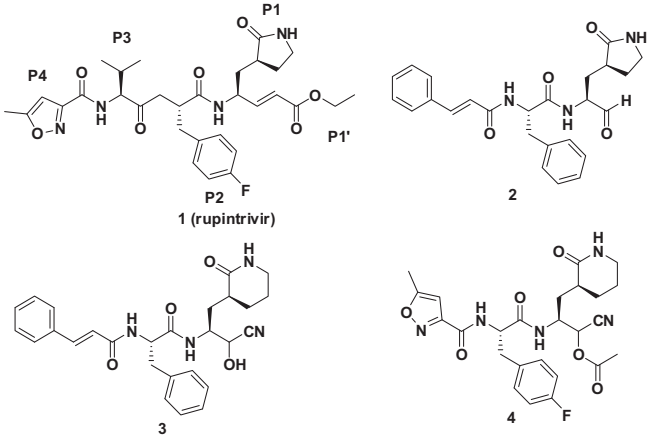

Figure 1. Structures of EV71 $3C^{pro}$ inhibitors.

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0960-894X/

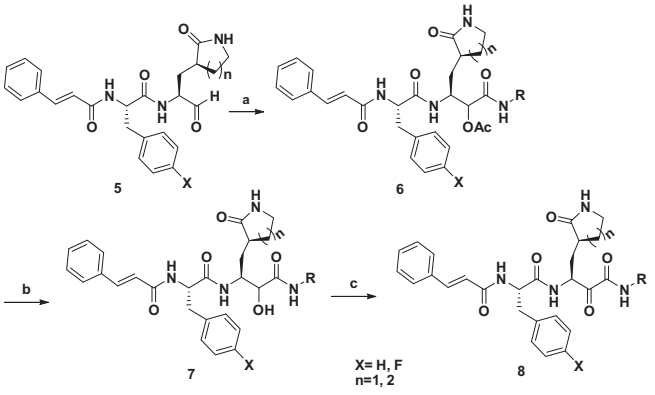
aldehyde as the electrophile exhibited even better activity (IC_{50} < 0.5 μM).[13] In recent years, our lab discovered a series of cyanohydrin derivatives as potent and selective inhibitors of EV71 $3C^{pro}$ (e.g., **3**, **4**).[14] More importantly, we obtained the co-crystal structure of **3**/EV71 $3C^{pro}$, which revealed the interactions between the cyanohydrin group and $3C^{pro}$. α-Keto amide, a mild electrophilic functional moiety with good druggability, has been used widely in cysteine and serine protease inhibitors, for example boceprevir for HCV NS3/4A inhibitor.[15] Additionally, α-keto amides offer an opportunity to extend interaction with S1′ pocket and to study structure–activity relationship (SAR) of P1′. Herein a series of α-keto amides as EV71 $3C^{pro}$ inhibitors were reported and SAR was discussed.

Synthesis of α-keto amide from aldehyde via cyanohydrin was reported.[16] However, the toxic potassium cyanide was used in the reported approach and the overall yield was only as low as 20% due to its lengthy steps. On the basis of previously reported methods,[17] an improved synthesis of α-keto amides was accomplished via Passerini reaction. As illustrated in Scheme 1, aldehyde **5**, which was synthesized according to the literature,[14] was treated with isocyanide and acetic acid to give ester **6**. Then alcohol **7**, obtained by removal of acetyl group of **6** under basic condition, was oxidized with Dess–Martin periodinane to give α-keto amide **8**. As a result of shorter steps, high conversion ratio and only one purification process, the overall yield reached as high as 70%.

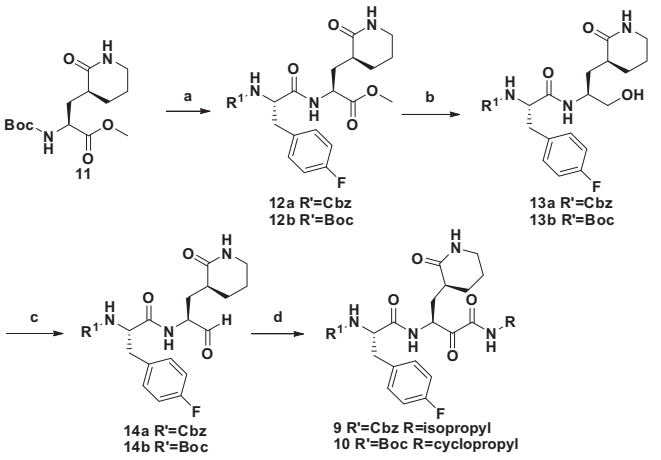
Synthesis of α-keto amides **9** and **10** started from key intermediate **11** which was prepared similarly to literature.[16] As illustrated in Scheme 2, removal of the Boc group of **11** with TFA followed by an amide bond formation using EDCI as coupling reagent resulted in **12**. Alcohol **13**, obtained by the reduction of ester **12** with $NaBH_4$, was finally oxidized to aldehyde **14** with Dess–Martin periodinane. α-Keto amides **9** and **10** were obtained using the method similarly to **8**.

The inhibitory activities (IC_{50}) of the α-keto amide inhibitors against EV71 $3C^{pro}$ were studied using a fluorescence resonance energy transfer (FRET)-based enzyme assay.[18] The anti-EV71 activities of these inhibitors were evaluated by an in vitro cell-based assay with the EV71 replicon cell system,[14a,19] and the results were expressed as EC_{50} values for antiviral activity and CC_{50} values for cytotoxicity.

With aldehyde **2** (IC_{50} = 3.81 ± 0.19 μM, EC_{50} = 3.07 ± 0.20 μM) and **5** (IC_{50} = 0.54 ± 0.02 μM, EC_{50} = 0.26 ± 0.07 μM) as Ref. 16, the antiviral activities of EV71 $3C^{pro}$ inhibitors containing P1 modifications ((*S*)-γ-lactam ring vs (*S*)-δ-lactam ring) were compared (Table 1). Overall, the activities of α-keto amide inhibitors were less potent than that of aldehyde inhibitors, probably due to the mild electrophilic reactivity of α-keto amide. The biological



Scheme 1. Improved synthesis of α-keto amide inhibitors. Reagents and conditions: (a) RNC, AcOH, DCM, rt; (b) LiOH, MeOH/H_2O, rt; (c) Dess–Martin periodinane, DCM, rt, 70–75% from **5** to **8**.



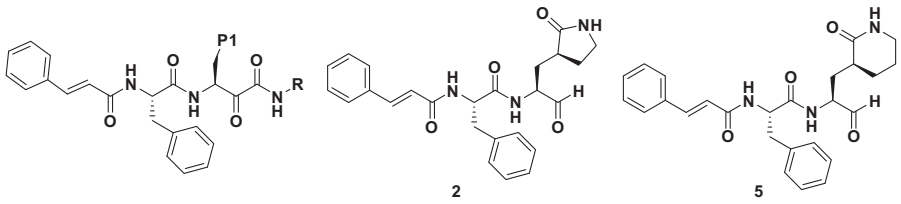
Scheme 2. Synthesis of α-keto amide inhibitors **9** and **10**. Reagents and conditions: (a) (i) TFA, DCM, rt; (ii) Boc-L-Phe(4-F)-OH or Cbz-L-Phe(4-F)-OH, EDCI, HOBt, TEA, DCM; 65–74%; (b) $NaBH_4$, MeOH, rt, 85–89%; (c) Dess–Martin periodinane, DCM, rt, 90–92%; (d) (i) RNC, AcOH, DCM, rt; (ii) LiOH, MeOH/H_2O, rt; (iii) Dess–Martin periodinane, DCM, rt; 72–75%.

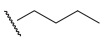
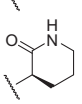
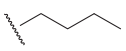
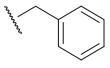
activities indicated that P1 (*S*)-δ-lactam ring bearing analogs **8f–8j** presented approximately 2- to 6-fold better activities than **8a–8e** containing (*S*)-γ-lactam ring at P1 position in both the enzyme and cellular assays. This result was consistent with the activity comparison between aldehyde **2** and **5**. It was clear that replacement of (*S*)-γ-lactam ring by (*S*)-δ-lactam ring could improve the potency of inhibitors against EV71 $3C^{pro}$. Additionally, low toxicity (CC_{50} > 100 μM) was observed for all the α-keto amide inhibitors. Hence, the (*S*)-δ-lactam ring as standard P1 residue for α-keto amide inhibitors were investigated in the following studies.

In order to explore the SAR of P1′, various α-keto amide inhibitors containing different groups were synthesized and evaluated (Tables 1 and 2). The results showed that most α-keto amides with short terminal chains (less than 5 carbons) at P1′ gave satisfactory activities, with IC_{50} values from 1.34 ± 0.33 μM to 8.21 ± 1.96 μM and EC_{50} values from 1.66 ± 0.45 μM to 11.6 ± 3.96 μM. α-Ketoamides **8p**, **8q** and **8r** with long alkyl chains showed dissatisfactory activities (IC_{50} > 20 μM) against EV71 $3C^{pro}$. Moreover, α-keto amides with small branched alkyls at P1′ showed improved activities, compared with the corresponding α-keto amides with straight-chain alkyls. For example, **8f** and **8m** with isopropyl and cyclopropyl displayed 2–4 fold better activities than **8l**, with IC_{50} value of 1.34 ± 0.33 μM, 3.32 ± 0.43 μM and 6.22 ± 1.07 μM, respectively. Similar results could be found in comparison of the activities of inhibitors **8g** (IC_{50} = 8.21 ± 1.96 μM) and **8n** (IC_{50} = 5.07 ± 0.89 μM). However, **8o** containing *t*-Bu at P1′, exhibited poor anti-EV71 $3C^{pro}$ activity (IC_{50} > 20 μM), suggesting that steric effect needed to be considered at P1′. Additionally, for inhibitors **8i**, **8s** and **8t**, the presence of phenyl group and substituted phenyl groups were apparently responsible for the loss of activity (IC_{50} > 20 μM). Structurewise, the entrance of S1′ pocket in EV71 $3C^{pro}$ was rather narrow,[10a] which may result in the poor tolerance of sterically rigid and bulky phenyl groups and *t*-Bu group. However, it was interesting to find that **8j** and **8u** containing aryl methylene groups displayed satisfactory anti-EV71 $3C^{pro}$ activities (IC_{50} = 7.83 ± 1.23 μM and 6.30 ± 1.12 μM, respectively). The addition of methylene group made the aryl moieties more flexible, which led to aryl moieties better fit to the S1′ pocket. Moreover, all the α-keto amide inhibitors were low toxic, with CC_{50} values >100 μM.

With the *para*-fluoro benzyl group instead of benzyl group at P2, **8v** (IC_{50} = 1.32 ± 0.26 μM) showed comparable activity

Table 1
The structures of α-keto amide inhibitors and their enzyme inhibitory activities, antiviral activities and cytotoxicities as EV71 $3C^{pro}$ inhibitors



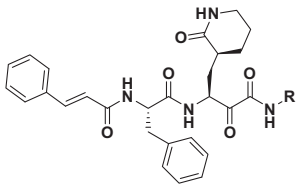
Compd no.	P1	R	IC_{50}[a] (μM)	EC_{50}[a] (μM)	CC_{50} (μM)
2	—	—	3.81 ± 0.19	3.07 ± 0.20	>100
5	—	—	0.54 ± 0.02	0.26 ± 0.07	>100
8a			8.75 ± 1.65	6.45 ± 1.78	>100
8b			17.6 ± 4.80	15.5 ± 3.48	>100
8c			12.7 ± 2.95	8.12 ± 1.89	>100
8d			>20	>20	>100
8e			>20	>20	>100
8f			1.34 ± 0.33	1.66 ± 0.45	>100
8g			8.21 ± 1.96	11.6 ± 3.96	>100
8h			4.86 ± 0.58	2.63 ± 0.44	>100
8i			>20	>20	>100
8j			7.83 ± 1.23	5.42 ± 1.68	>100

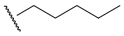
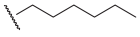
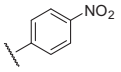
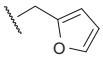
[a] Measurements of enzymatic and in vitro activity were performed in triplicate and represent the mean ± SD of at least three experiment sets.

compared with **8f** (IC_{50} = 1.34 ± 0.33 μM) and **8w**–**8y** (IC_{50} = 1.88 ± 0.35 μM, 1.52 ± 0.31 μM and 3.71 ± 0.55 μM, respectively) exhibited increased potency by 2–3 fold. More importantly, **8v**, **8w**, **8x** represented excellent antiviral activities in cellular system (EC_{50} = 1.12 ± 0.23 μM, 1.08 ± 0.25 μM and1.55 ± 0.33 μM, respectively). Additionally, in our previous report of the cyanohydrin inhibitors,[16] the introduction of *para*-fluoro on benzyl group was considered to play an important role on electron distribution of the S2 pocket, which was consistent with the increased activities of **8v**–**8y**. With the cap (P3) replaced by carbobenzoxy (Cbz) moiety or *t*-butyloxycarbonyl (Boc) moiety, **9** and **10** (IC_{50} = 1.42 ± 0.38 μM, 1.78 ± 0.45 μM, respectively) exhibited comparable activity with **8v** and **8x**. It may suggest that variation of P3 has less effect on activity compared to P1′, P1 and P2 (Table 3).

In order to study the binding modes of α-keto amide inhibitors bound to EV71 $3C^{pro}$, docking study was performed using Autodock software. Similar to rupintivir-liganded protease complex,[10a] both **8a** and **8f** fill the pockets of active sites of EV71 $3C^{pro}$ (Fig. 2). According to the docking models, ring expansion made (*S*)-δ-lactam ring of **8f** occupy S1 pocket of EV71 $3C^{pro}$ more than (*S*)-γ-lactam ring of **8a**, resulting in the activity increase of **8f** compared to **8a**. Additionally, moieties with rigid steric hindrance (phenyl or tertiary butyl) and moieties with long alkyl chains (hexyl, pentyl, and so on) were not tolerated at S1′ pocket (models are not shown), which was consistent with experimental results of α-keto amides. Then the most potent α-keto amide inhibitor **8v** was docked into EV71 $3C^{pro}$ and the interaction pattern was illustrated (Fig. 3). The distance between anchoring group (carbonyl) of **8v** and thiol of Cys147 was 3.6 Å. Moreover, the carbonyl formed hydrogen-bonding interaction (3.0 Å) with the backbone of Gly145 making the anchoring group more positive, hence increasing the reactivity of **8v** with thiol of Cys147. Additionally, carbonyl of P1′ fragment engaged in hydrogen-bonding interaction with the side-chain of His40 (3.1 Å). The S1 pocket is formed by Thr142, His161, Gly163 and Gly164 and interacted with P1 residue of **8v** through multiple hydrogen-bonding. The carbonyl of (*S*)-δ-lactam ring was

Table 2
The structures of α-keto amide inhibitors with P1′ modifications and their enzyme inhibitory activities, antiviral activities and cytotoxicities as EV71 $3C^{pro}$ inhibitors



Compd no.	R	IC_{50}[a] (μM)	EC_{50}[a] (μM)	CC_{50} (μM)
8k		4.08 ± 0.78	9.59 ± 2.89	>100
8l		6.22 ± 1.07	4.59 ± 1.02	>100
8m		3.32 ± 0.43	3.57 ± 0.79	>100
8n		5.07 ± 0.89	11.01 ± 1.98	>100
8o		>20	>20	>100
8p		>20	>20	>100
8q		>20	>20	>100
8r		>20	>20	>100
8s		>20	>20	>100
8t		>20	>20	>100
8u		6.30 ± 1.12	4.50 ± 1.18	>100

[a] Measurements of enzymatic and in vitro activity were performed in triplicate and represent the mean ± SD of at least three experiment sets.

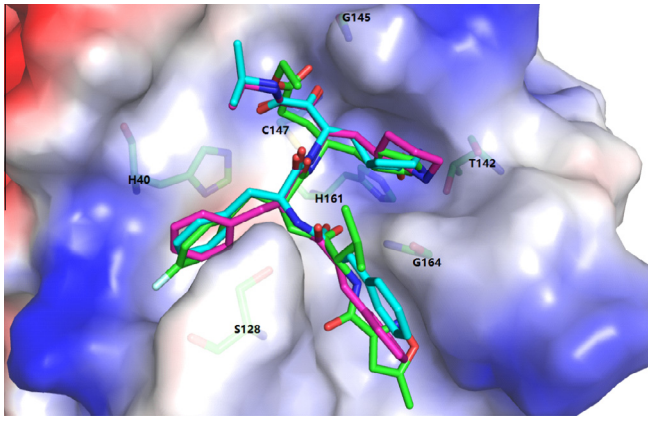


Figure 2. Comparison between the docking models of α-keto amide **8a** (cyan), **8f** (purple) bound to EV71 $3C^{pro}$ and co-crystal structure of rupintrivir (green)/EV71 $3C^{pro}$ (PDB code: 4GHT).

employed in the hydrogen-bonding interactions with the side-chains of Thr142 and His161 (3.3 Å and 3.5 Å, respectively). And the amide nitrogen of (*S*)-δ-lactam ring donated hydrogen bonds to the backbone of Thr142 and Gly163 (3.0 Å and 3.2 Å, respectively). The docking model indicated that *para*-fluoro benzyl played a significant role on electron distribution of the S2 pocket, thus enhancing the interaction with positively charged Arg39. Furthermore, the benzene ring of **8v** at P2 interacted with His40 by π–π edge stacking, approximately 4.4 Å. The amide, combining P2 and P3 fragments, was involved in the hydrogen-bonding interactions with surrounding residues (Gly164 and

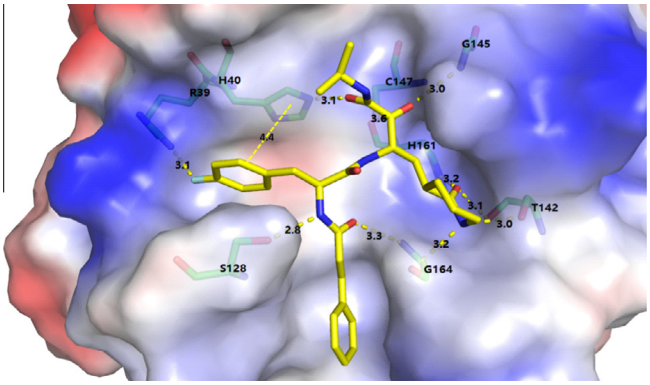
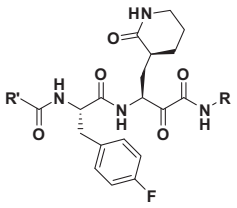
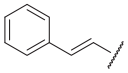
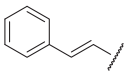
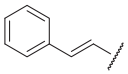
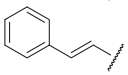
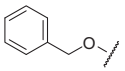


Figure 3. Docking model of **8v** bound to EV71 $3C^{pro}$.

Table 3
The structures of α-keto amide inhibitors with P1′ and P3 modifications and their enzyme inhibitory activities, antiviral activities and cytotoxicities as EV71 $3C^{pro}$ inhibitors



Compd no.	R	R′	IC_{50}[a] (μM)	EC_{50}[a] (μM)	CC_{50} (μM)
8v			1.32 ± 0.26	1.12 ± 0.23	>100
8w			1.88 ± 0.35	1.08 ± 0.25	>100
8x			1.52 ± 0.31	1.55 ± 0.33	>100
8y			3.71 ± 0.55	2.78 ± 0.43	>100
9			1.42 ± 0.38	1.32 ± 0.33	>100
10			1.78 ± 0.45	1.68 ± 0.41	>100

[a] Measurements of enzymatic and in vitro activity were performed in triplicate and represent the mean ± SD of at least three experiment sets.

Ser128) of EV71 $3C^{pro}$. At last, the wide and deep S3 pocket is consist of various hydrophobic residues, such as Tyr122, Leu125, Leu127, Gly163 and Phe170. Hydrophobic moieties (styryl, Boc, Cbz, etc.) were well tolerated and interact with S3 pocket residues by hydrophobic interactions.

In summary, a series of α-keto amides as potent inhibitors of EV71 $3C^{pro}$ were described. Inhibitors **8v**, **8w** and **8x** were considered to be the best α-keto amide inhibitors (IC_{50} = 1.32 ± 0.26 μM, 1.88 ± 0.35 μM and 1.52 ± 0.31 μM, respectively). As expected, favorable CC_{50} values (CC_{50} > 100 μM) were observed for all of α-keto amide inhibitors in the in vitro cytotoxicity assay. SAR study indicated that small moieties were primely tolerated at P1′ and the introduction of *para*-fluoro benzyl at P2 notably improved the potency of inhibitor. Therefore, α-keto amide represents a good choice as a warhead for EV71 $3C^{pro}$ inhibitor.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.02.039. These data include MOL files and InChiKeys of the most important compounds described in this article.

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