Supporting information for:

Extended structure-activity relationship and pharmacokinetic evaluation of (4-quinolinoyl)-glycyl-2-cyanopyrrolidine inhibitors of fibroblast activation protein (FAP)

Koen Jansen, † Leen Heirbaut, † Jonathan D. Cheng, † Jurgen Joossens, † Oxana Ryabtsova, † Paul Cos, † Louis Maes, § Anne-Marie Lambeir, †† Ingrid De Meester, †† Koen Augustyns, † Pieter Van der Veken.*†

† Medicinal Chemistry (UAMC), Department of Pharmaceutical Sciences, University of Antwerp (UA), Universiteitsplein 1, B-2610 Antwerp, Belgium; † Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia PA-19111-2497, USA; § Laboratory of Microbiology, Parasitology and Hygiene (LMPH), Departments of Pharmaceutical and Biomedical Sciences, University of Antwerp (UA), Universiteitsplein 1, B-2610 Antwerp, Belgium; †† Medical Biochemistry, Department of Pharmaceutical Sciences, University of Antwerp (UA), Universiteitsplein 1, B-2610 Antwerp, Belgium

*corresponding author

Table of Contents:

I. Synthetic procedures and analytical data for intermediates .............................................. S2

II. Enzymatic assay data

II.1 Assay conditions for IC50 determinations................................................................. S15

II.2 Ex vivo Enzyme activity assays................................................................................... S17

III. In vivo PK experiments................................................................................................. S18

IV. In vitro PK experiments............................................................................................... S20

V. References..................................................................................................................... S24
I. Synthetic procedures and analytical data for intermediates

This part contains detailed procedures and analytical information for the intermediates specified below, the synthesis of which was not included in the manuscript text or described earlier in literature:

I.1 (S)-4,4-difluoro-1-glycylpyrrolidine-2-carbonitrile 4-methylbenzenesulfonate (S8). This compound is used as a building block for the synthesis of final compounds 60-63. The further synthesis of these final products is given in Scheme 2 of the manuscript.

I.2 N-methyl-1-(quinolin-4-yl)methanamine (S9) and 2-methoxy-N-(quinolin-4-ylmethyl)ethanamine (S10). These intermediates were used as building blocks for the synthesis of final compounds 21 and 22. The further synthesis of these final products is given in Scheme 1 of the manuscript.

I.3 2-Phenylisonicotinic acid (S11), 2-(3,4-dimethoxyphenyl)isonicotinic acid (S12) and 2-(4-cyanophenyl)isonicotinic acid (S13). These intermediates were used in the synthesis of final compounds 33-35. The further synthesis of these final products is given in Scheme 1 of the manuscript.

I.4 (2S,4R)-4-fluoropyrrolidine-2-carbonitrile 4-methylbenzenesulfonate (S15) and (2S,4S)-4-fluoropyrrolidine-2-carbonitrile 4-methylbenzenesulfonate (S16). As described in the manuscript, these compounds were originally synthesized by us using literature procedures (reference 29 of the manuscript). An optimized protocol that renders substantially higher yields is described here. These intermediates were used in the synthesis of final compounds 58 and 59. The further synthesis of these final products is given in Scheme 2 of the manuscript.

I.5 2-(4-Fluorophenyl)-7-methyl-3H-imidazo[4,5-b]pyridine (S17), 2-(4-fluorophenyl)-7H-imidazo[4,5-b]pyridine-7-carboxylic acid (S18), 2-(4-methoxyphenyl)-7-methyl-3H-imidazo[4,5-b]pyridine (S19) and 2-(4-methoxyphenyl)-7H-imidazo[4,5-b]pyridine-7-carboxylic acid (S20). Intermediates S17 and S18 were used as building blocks for final compound 48. Intermediates S19 and S20 were used for the synthesis of final compound 49. The further synthesis of these final products is given in Scheme 1 of the manuscript.

I.6 4-Bromo-1,7-naphthyridine (S21), 1,7-naphthyridine-4-carbonitrile (S22) and 1,7-naphthyridine-4-carboxylic acid (S23). These intermediates were used in the synthesis of final compound 51. The further synthesis of this final product is given in Scheme 1 of the manuscript.
I.7 7-Phenylquinoline-4-carboxylic acid (S24) and 7-(phenylamino)quinoline-4-carboxylic acid (S25). These intermediates were used in the synthesis of final compounds 54 and 55. The further synthesis of these final products is given in Scheme 1 of the manuscript.

I.8 4-Methylisatin (S26), 6-methylisatin (S27), 5-methylquinoline-2,4-dicarboxylic acid (S28), 7-methylquinoline-2,4-dicarboxylic acid (S29), 5-methylquinoline-4-carboxylic acid (S30) and 7-methylquinoline-4-carboxylic acid (S31). These intermediates were used in the synthesis of final compounds 52 and 53. The further synthesis of these final products is given in Scheme 1 of the manuscript.

I.9 5-Phenylquinoline-4-carboxylic acid (S32) and 5-methoxyquinoline-4-carboxylic acid (S33). These intermediates were used in the synthesis of final compounds 56 and 57. The further synthesis of these final products is given in Scheme 1 of the manuscript.

I.10 2-Amino-1-(pyrrolidin-1-yl)ethanone hydrochloride (S34) and (S)-tert-butyl 2-(2-chloroacetyl)pyrrolidine-1-carboxylate (S35). These intermediates were used in the synthesis of final compounds 64 and 67. The further synthesis of these final products is given in Scheme 2 of the manuscript.

I.1 (S)-4,4-difluoro-1-glycylpyrrolidine-2-carbonitrile 4-methylbenzenesulfonate (S8)

Scheme S1: synthesis of (S)-4,4-difluoro-1-glycylpyrrolidine-2-carbonitrile 4-methylbenzenesulfonate (S8)

\[ \text{Scheme S1: synthesis of (S)-4,4-difluoro-1-glycylpyrrolidine-2-carbonitrile 4-methylbenzenesulfonate (S8)} \]

\[ \text{[S3]} \]
(S)-1-tert-butyl 2-methyl 4-oxopyrroolidine-1,2-dicarboxylate (S1)

1,3,5-Trichloro-1,3,5-triazinane-2,4,6-trione (3.78 g, 16.27 mmol) was added to a cooled (0°C) solution of (S)-1-tert-butyl 2-methyl 4-oxopyrroolidine-1,2-dicarboxylate (3.8 g, 15.49 mmol) in DCM (25 ml), followed by the addition of catalytic TEMPO (0.024 g, 0.155 mmol). After 5 min the mixture was allowed to reach room temperature, stirred for another 30 minutes and filtrated over Celite. The organic layer was washed with 20 ml saturated potassium carbonate solution, sodium thiosulfate, brine, dried over anhydrous sodium sulfate, filtrated and evaporated. The crude compound 1 was used without further purification.

\(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta\) 4.77 (dd, \(J = 36.8, 8\) Hz, 1H), 3.88 (br s, 2H), 3.75 (s, 3H), 2.90 (s, 1H), 2.57 (dd, \(J = 18.8, 2.4, 1H\)), 1.46 (s, 9H); MS (ESI) m/z 376.2 [M + MeOH + H]^+; (2.77 g, 74%)

( S)-1-tert-butyl 2-methyl 4,4-difluoropyrroolidine-1,2-dicarboxylate (S2)

A solution of the (S)-1-tert-butyl 2-methyl 4-oxopyrroolidine-1,2-dicarboxylate (0.23 g, 0.946 mmol), obtained from step 1, in DCM (3 ml), in a 25-ml flask equipped under nitrogen and stirring bar, was treated with a solution of diethylaminosulfur trifluoride (0.197 ml, 1.607 mmol) in DCM (2 ml) at room temperature. Ethanol (0.011 ml, 0.189 mmol) was added and the mixture was stirred for 18h at room temperature. The solution was poured into saturated sodium bicarbonate and after CO\(_2\) evolution ceased it was extracted into DCM (3 x 15 ml), dried (Na\(_2\)SO\(_4\)), filtered, and evaporated in vacuo. Chromatography on silica gel in DCM afforded a yellowish oil.

\(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta\) 4.55 - 4.45 (m, 1H), 3.90 - 3.60 (m, 2H), 3.75 (s, 3H), 2.81 - 2.61 (m, 1H), 2.45 (dq, \(J = 13.6, 5.2\) Hz, 1H), 1.44 (br s, 9H); MS (ESI) m/z 266.1 [M + H]^+; (0.150 g, 61%)

( S)-1-(tert-butoxycarbonyl)-4,4-difluoropyrroolidine-2-carboxylic acid (S3)

(S)-1-tert-butyl 2-methyl 4,4-difluoropyrroolidine-1,2-dicarboxylate (1.51 g, 5.69 mmol), obtained from step 2, was dissolved in 6 ml of 1M potassium hydroxide solution. The solution was stirred overnight. The mixture was washed with ether, acidified, extracted with ethyl acetate, washed with brine, dried over sodium sulfate, filtered and evaporated to yield slightly brownish crystals. The crude mixture was used without further purification.

\(^1\)H NMR (400 MHz, DMSO-\(d_6\)): \(\delta\) 13.0 (br s, 1H), 4.37 - 4.31 (m, 1H), 3.77 - 3.65 (m, 2H), 2.98 - 2.78 (m, 1H), 2.48- 2.32 (m, 1H), 1.38 (s, 9H); UPLC I R, 1.51 min, m/z 252.5 [M+H]^+ (90%); (1.23 g, 86%)

(S)-tert-butyl 2-carbamoyl-4,4-difluoropyrroolidine-1-carboxylate (S4)

[S4]
In a 50 ml round-bottomed flask (S)-1-(tert-butoxycarbonyl)-4,4-difluoropyrrolidine-2-carboxylic acid (1.6 g, 6.37 mmol), obtained from step 4, was dissolved in 10 ml of dichloromethane at 15°C. Then 1-hydroxypyrrrolidine-2,5-dione (0.806 g, 7.0 mmol) was added. To the formed suspension N,N’-dicyclohexylcarbodiimide (1.445 g, 7.0 mmol) was added at vigorous stirring. In a few seconds a cloudy white suspension forms. The mixture was allowed to reach RT and stirred for 30 min, followed by the addition of 7N ammonia in methanol (2.0 ml, 14 mmol) and stirring for another 20 min. Before evaporation of volatile components 5 g of Celite was added to the flask. Cold ethyl acetate was added to the residue and filtered over Celite. The filtrate was washed with saturated sodium bicarbonate. The formed slightly yellowish crystals were used without further purification.

\(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta\) 6.77 (br s, 1H), 5.54 (br s, 1H), 4.52 (br s, 1H), 4.00 - 3.80 (m, 1H), 3.78 - 3.56 (m, 1H), 3.02 - 2.82 (m, 1H), 2.77 - 2.44 (m, 1H), 1.45 (s, 9H); LC-MS (A) R\(_t\) 1.34 min, m/z 251.5 [M+H]\(^+\) (89%); MS (ESI) m/z 251.2 [M + H]\(^+\); (1.15 g, 72%)

**(S)-4,4-difluoropyrrolidine-2-carboxamide trifluoroacetate (S5)**

9.54 ml of trifluoroacetic acid was added to a solution of (S)-tert-butyl 2-carbamoyl-4,4-difluoropyrrolidine-1-carboxylate (1.25 g, 5 mmol) in 10 ml of dichloromethane. The solution was stirred for 1 h before evaporation. The residue was washed with ether to yield white crystals.

\(^1\)H NMR (400 MHz, DMSO-\(d_6\)): \(\delta\) 10.01 (br s, 2H), 8.07 (s, 1H), 7.82 (s, 1H), 4.46 (t, J = 8.59 Hz, 1H), 3.71 (d, J = 19.54, 7.48 Hz, 2H), 3.00 - 2.86 (m, 1H), 2.61 - 2.42 (m, 1H); MS (ESI) m/z 155.2 [M + H]\(^+\); (0.84 g, 90%)

**((S)-tert-butyl 2-(2-carbamoyl-4,4-difluoropyrrolidin-1-yl)-2-oxoethylcarbamate (S6)**

HATU (12.47 g, 32.8 mmol) was dissolved in 20 ml DMF and added to a solution of 2-(tert-butoxycarbonylamino)acetic acid (5.75 g, 32.8 mmol), and DIPEA (5.43 ml, 32.65 mmol) in 30 ml DCM. After 10 min a solution of (S)-4,4-difluoropyrrolidine-2-carboxamide hydrochloride (5.1 g, 27.3 mmol) and DIPEA (9.1 ml, 54.4 mmol) in 40 ml DCM was added. After 3 h the cloudy mixture was filtered of. The filtrate was cooled and filtrated again. The combined residues were washed with DCM and water and recrystallized from ethyl acetate.

\(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) 7.73 - 7.13 (m, 2H), 6.89 (br tr, J = 5.6 Hz, 1H), 4.46 (dd, J = 9.72, 4.13 Hz, 1H), 4.09 (dd, J = 25.40, 12.76 Hz, 1H), 3.96 (dd, J = 24.58, 11.16 Hz, 1H), 3.81 (dd, J = 17.2, 5.68 Hz, 1H), 3.71 (dd, J = 17.2, 5.32 Hz, 1H), 2.78 - 2.65 (m, 1H), 2.43 - 2.30 (m, 1H), 1.39 (s, 9H); LC-MS (A) R\(_t\) 1.28 min, m/z 308.5 [M+H]\(^+\) (94%); MS (ESI) m/z 308.1 [M + H]\(^+\); (8.39 g, 77%)

[S5]
(S)-tert-butyl 2-(2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethylcarbamate (S7)

In a 50 ml round-bottomed flask (S)-tert-butyl 2-(2-carbamoyl-4,4-difluoropyrrolidin-1-yl)-2-oxoethylcarbamate (0.720 g, 2.343 mmol) was dissolved in dry THF at -15°C. Then pyridine was added, followed by the dropwise addition of the solution of 2,2,2-trifluoroacetic anhydride (0.094 ml, 0.664 mmol) in 5 ml of DCM after the complete addition, the mixture was allowed to reach RT. The reaction mixture was stirred for 90 minutes. The reaction mixture was washed with 1 M solution of aqueous solution of hydrochloric acid. Then the organic layer was washed three times with saturated sodium bicarbonate, brine, dried over sodium sulfate and evaporated. The crude mixture was purified using column chromatography (hexane-ethyl acetate 2-3) yielding a yellowish oil.

1H NMR (400 MHz, CDCl$_3$) δ 5.29 (br s, 1H), 5.00 - 4.94 (m, 1H), 4.04 - 3.80 (m, 4H), 2.83 - 2.70 (m, 2H), 1.45 (s, 9H); LC-MS (A) R$_t$ 1.52 min, m/z 290.6 [M+H]$^+$ (94%); MS (ESI) m/z 290.1 [M+H]$^+$ (0.678 g, 70%)

N-methyl-1-(quinolin-4-yl)methanamine (S9) and 2-methoxy-N-(quinolin-4-yl)methyl)ethanamine (S10).

(S)-1-(2-aminoacetyl)-4,4-difluoropyrrolidine-2-carbonitrile 4-methylbenzenesulfonate (S8)

p-Toluenesulfonic acid monohydrate (3.59 g, 18.87 mmol) was added to a cooled (0°C) solution of (S)-tert-butyl 2-(2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethylcarbamate (3.9 g, 13.48 mmol) in 35 ml of acetonitrile. The mixture was slowly warmed to room temperature and stirred for 24 h. The residue was evaporated to dryness and washed with cold ether and cold ethyl acetate and dried again to give off-white crystals.

1H NMR (400 MHz, D$_2$O): (5/1 mixture of trans/cis amide rotamers) δ 7.47 (d, J = 7.8 Hz, 2H), 7.11 (d, J = 7.8 Hz, 2H), 5.37 (d, J = 8.4 Hz, 0.2H) 5.17 (dd, J = 8.4, 4.1 Hz, 0.8H), 4.24 - 4.03 (m, 2H), 4.01 (s, 2H), 3.05-2.84 (m, 2H), 2.29 (s, 3H) MS (ESI) m/z: 190.2 [M+H]$^+$; (4.3 g, 90%)

N-methyl-1-(quinolin-4-yl)methanamine (S9)

Quinoline-4-carbaldehyde (0.5 g, 3.18 mmol) was dissolved in 30 ml of dry EtOH and added to methanamine (0.618 ml, 15.91 mmol) also dissolved in 30 ml of dry EtOH. A few drops of acetic acid were added and the resulting solution was stirred for 2 h, then sodium borohydride (0.554 g, 14.63 mmol) was added portionwise. After 2 h at room temperature, the solvent was evaporated to dryness. The resulting residue was treated with water and repeatedly extracted with
dichloromethane (3 × 50 ml). The organic phase was then dried with anhydrous sodium sulfate, and the solvent was evaporated to yield the free amine as a yellowish oil. (0.320 g, 58%)

\(^1\)H NMR (CDCl\textsubscript{3}, 400 MHz) δ 8.60 (d, J = 4.47 Hz, 1H), 7.92 (dd, J = 7.7, 0.7 Hz, 1H), 7.77 (dd, J = 8.5, 1.3 Hz, 1H), 7.45 (ddd, J = 8.4, 6.9, 1.5 Hz, 1H), 7.29 (ddd, J = 8.3, 6.9, 1.4 Hz, 1H), 7.12 (d, J = 4.3 Hz, 1H), 3.87 (s, 2H), 3.28 (s, 3H), 2.26 (br s, 1H); UPLC (ESI) \( R_t \) 0.22, m/z 173.3 [M+H]\(^+\) (94%);

2-methoxy-N-(quinolin-4-ylmethyl)ethanamine (S10)

The title compound was made in a similar manner as S9.

\(^1\)H NMR (CDCl\textsubscript{3}, 400 MHz) δ 8.74 (d, J = 4.39, 1H), 8.03 (dd, J = 8.4, 1.3 Hz, 1H), 7.58 (dd, J = 8.4, 6.9, 1.5 Hz, 1H), 7.93 (dd, J = 8.5, 1.4 Hz, 1H), 7.43 (ddd, J = 8.3, 6.9, 1.4 Hz, 1H), 7.33 (d, J = 4.4 Hz, 1H), 4.14 (s, 2H), 3.44 (t, J = 5.1, 2H), 3.26 (s, 3H), 2.79 (t, J = 5.1 Hz, 2H), 2.26 (br s, 1H); UPLC (ESI) \( R_t \) 0.20, m/z 217.3 [M+H]\(^+\) (95%); (0.210 g, 43%)

I.3 2-Phenylisonicotinic acid (S11), 2-(3,4-dimethoxyphenyl)isonicotinic acid (S12) and 2-(4-cyanophenyl)isonicotinic acid (S13).

2-phenylisonicotinic acid (S11)

The title compound was prepared in a similar manner as S13.

\(^1\)H NMR (400 MHz, DMSO-d\textsubscript{6}) δ 13.62 (br s, 1H), 8.93 – 8.79 (dd, J = 5.1, 0.8 Hz, 1H), 8.35 – 8.23 (t, J = 1.2 Hz, 1H), 8.19 – 8.05 (m, 2H), 7.86 – 7.71 (dd, J = 4.9, 1.5 Hz, 1H), 7.60 – 7.44 (m, 3H); UPLC (ESI) \( R_t \) 1.35 min, m/z 200.5 [M+H]\(^+\) (100%); (0.1 g, 48%)

2-(3,4-dimethoxyphenyl)isonicotinic acid (S12)

The title compound was prepared in a similar manner as S13.

\(^1\)H NMR (400 MHz, DMSO-d\textsubscript{6}) δ 13.73 (s, 1H), 8.80 (dd, J = 5.0, 0.9 Hz, 1H), 8.25 (t, J = 1.2 Hz, 1H), 7.70 (ddd, J = 10.5, 5.5, 2.1 Hz, 3H), 7.08 (d, J = 8.4 Hz, 1H), 3.86 (s, 3H), 3.83 (s, 3H); UPLC (ESI) \( R_t \) 1.21 min, m/z 260.5 [M+H]\(^+\) (96%); (0.13 g, 49%)

2-(4-cyanophenyl)isonicotinic acid (S13)

2-bromoisonicotinic acid (0.210 g, 1.040 mmol) was dissolved in DME (8 ml) under nitrogen and the solvent was degassed in the ultrasonic bath under nitrogen. Tetrakis(triphenylphosphine)palladium(0) (0.060 g, 0.052 mmol) was added, the resulting reaction mixture was stirred for 15 min. Degassed aqueous potassium carbonate (4.16 ml, 8.32 mmol) and 4-cyanophenylboronic acid (0.206 g, 1.403 mmol) were added subsequently. The resulting reaction
mixture was refluxed at 95°C for 18 h and then cooled to rt. After filtration over Celite and flushing the filter with DME and 5% sodium bicarbonate solution, the reaction mixture was acidified to pH 3-4 the white precipitate was filtered off, washed with water and recrystallized from 2-methoxyethanol to give a white powder. (0.180 g, 73%)

1H NMR (400 MHz, DMSO-\textit{d}6) \(\delta\) 13.92 (br s, 1H), 8.92 (d, \(J = 4.8\) Hz, 1H), 8.41 (t, \(J = 1.1\) Hz, 1H), 8.34 (d, \(J = 8.4\) Hz, 2H), 7.99 (d, \(J = 8.4\) Hz, 2H), 7.87 (dd, \(J = 5.2\), 1.3 Hz, 1H); UPLC I (ESI) \(R_t\) 1.45 min, m/z 225.4 [M+H]+ (98%).

I.4 (2S,4R)-4-fluoropyrrolidine-2-carbonitrile 4-methylbenzenesulfonate (S15) and (2S,4S)-4-fluoropyrrolidine-2-carbonitrile 4-methylbenzenesulfonate (S16).

(5)-\textit{tert}-butyl 4-(2-(2-cyanopyrrolidin-1-yl)-2-oxoethylcarbamoyl)phenylcarbamate (S14)

The target compound was made according to general procedure A.

1H NMR (400 MHz, DMSO-\textit{d}6) (trans-cis mixture of rotamers) \(\delta\) 9.63 (s, 1H), 8.56 (t, \(J = 5.7\) Hz, 1H), 7.79 (d, \(J = 8.8\) Hz, 2H), 7.53 (d, \(J = 8.8\) Hz, 2H), 5.26 (d, \(J = 4.9\) Hz, 0.1H), 4.75 (dd, \(J = 7.4\), 3.6 Hz, 0.9H), 4.28 - 4.17 (m, 0.2H), 4.11 (dd, \(J = 16.9\), 6.1 Hz, 0.9H), 4.03 (dd, \(J = 16.9\), 5.5 Hz, 0.9H), 3.74 - 3.65 (m, 1H), 3.64 - 3.56 (m, 1H), 2.28 - 1.94 (m, 4H), 1.48 (s, 9H); UPLC I (ESI) \(R_t\) 1.54 min, m/z 373.6 [M+H]+ (96%); (0.075 g, 67%)

\textbf{Scheme S2. Fluoroproline synthesis}

\begin{center}
\begin{tikzpicture}
  \node[draw,rectangle,inner sep=10pt] (A) {\textbf{F}};
  \node[draw,rectangle,inner sep=10pt] (B) [right of=A] {\textbf{H}};
  \node[draw,rectangle,inner sep=10pt] (C) [right of=B] {\textbf{O}};
  \node[draw,rectangle,inner sep=10pt] (D) [right of=C] {\textbf{Boc}};
  \node[draw,rectangle,inner sep=10pt] (E) [right of=D] {\textbf{NH}_2};
  \node[draw,rectangle,inner sep=10pt] (F) [right of=E] {\textbf{F}};
  \node[draw,rectangle,inner sep=10pt] (G) [right of=F] {\textbf{N}};
  \node[draw,rectangle,inner sep=10pt] (H) [right of=G] {\textbf{N}};
  \node[draw,rectangle,inner sep=10pt] (I) [right of=H] {\textbf{TsOH}};

  \path (A) edge [bend left=10] node [above] {a} (I);
  \path (I) edge [bend left=10] node [below] {b} (A);

\end{tikzpicture}
\end{center}

\textit{a}) TFAA, pyridine, THF, -15°C, (70-80%); \textit{b}) TsOH, MeCN, 80-86%

(2S,4R)-4-fluoropyrrolidine-2-carbonitrile 4-methylbenzenesulfonate (S15)

TFAA (0.154 ml, 1.089 mmol) was added to a solution of pyridine (0.561 ml, 6.93 mmol) and (2S,4R)-\textit{tert}-butyl 2-carbamoyl-4-fluoropyrrolidine-1-carboxylate (0.230 g, 0.990 mmol) in THF (5 ml) at 0°C. The mixture was stirred for 1 h, followed by evaporation, washing (3x) with 1 N HCl, sodium bicarbonate and brine. To give white crystals, which were used in the next reaction without further purification. (0.150 g, 70%)

\(p\)-Toluenesulfonic acid monohydrate (0.124 g, 0.653 mmol) was added to a cold (0°C) solution of (2S,4R)-\textit{tert}-butyl 2-cyano-4-fluoropyrrolidine-1-carboxylate (0.1 g, 0.467 mmol) in acetonitrile (about 0.4 M) the mixture was stirred at 0°C for 30 min and was then allowed to warm to rt and...
stirred for 24 h. The volatiles were evaporated and the residue washed with ethyl acetate to yield whitish crystals.

\(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) 7.48 (d, \(J = 8.0\) Hz, 2H), 7.12 (d, \(J = 8.0\) Hz, 2H), 5.59 - 5.41 (m, 1H), 4.87 (dd, \(J = 10.3, 7.2\) Hz, 1H), 3.60 (dd, \(J = 2.1, 1.1\) Hz, 1H), 3.57 - 3.47 (m, \(J = 3.3\) Hz, 1H), 2.79 - 2.67 (m, 1H), 2.58-250 (m, 1H), 2.44 (ddd, \(J = 14.6, 10.3, 4.2\) Hz, 1H), 2.30 (s, 3H); UPLC I (ESI) R\(_0\)0.20 min, m/z 115.4 [M+H\(^+\)] (98%)

\((25,4S)-4\text{-fluoropyrrolidine-2-carbonitrile 4-methylbenzenesulfonate (S16)}\)

The title compound was obtained as a white powder in a manner similar to the preparation of S15

\(^1\)H NMR (400 MHz, MeOD) \(\delta\) 7.71 (d, \(J = 8.2\) Hz, 2H), 7.24 (d, \(J = 7.9\) Hz, 2H), 5.62 - 5.44 (m, 1H), 4.97 - 4.91 (m, 1H), 3.83 - 3.72 (m, 1H), 3.60 - 3.46 (m, 1H), 2.84 - 2.72 (m, 1H), 2.72 - 2.65 (m, 1H), 2.37 (s, 3H). UPLC I (ESI) R\(_0\)0.19 min, m/z 115.4 [M+H\(^+\)] (93%); (0.460 g, 86%)

1.5 2-(4-Fluorophenyl)-7-methyl-3\(^H\)-imidazo[4,5-b]pyridine (S17), 2-(4-fluorophenyl)-3\(^H\)-imidazo[4,5-b]pyridine-7-carboxylic acid (S18), 2-(4-methoxyphenyl)-7-methyl-3\(^H\)-imidazo[4,5-b]pyridine (S19) and 2-(4-methoxyphenyl)-3\(^H\)-imidazo[4,5-b]pyridine-7-carboxylic acid (S20).

2-(4-Fluorophenyl)-7-methyl-3\(^H\)-imidazo[4,5-b]pyridine (S17) \(^3\)

A solution of 4-methyl-3-nitro-pyridin-2-ylamine (0.350 g, 2.286 mmol) and 4-fluorobenzaldehyde (0.240 ml, 2.286 mmol) in EtOH (4 ml) was treated with 1 M aq hydrosulfite (6.86 ml, 6.86 mmol). After heating the reaction mixture at 70°C for 24 h in a closed vial, it was cooled to rt and treated dropwise with 5 eq NH\(_4\)OH (2 ml). A precipitate was immediately formed which was then filtered, washed with water (2 x 15 ml) and dried under reduced pressure to afford the desired product in satisfactory purity. (0.140 g, 28%)

\(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) 13.46 (s, 1H), 8.34 – 8.24 (m, 2H), 8.17 (d, \(J = 5.3\) Hz, 1H), 7.48 – 7.36 (m, 2H), 7.07 (d, \(J = 4.9\) Hz, 1H), 2.60 (s, 3H); UPLC I (ESI) R\(_i\)1.21 min, m/z 228.5 [M+H\(^+\)] (94%)

2-(4-Fluorophenyl)-3\(^H\)-imidazo[4,5-b]pyridine-7-carboxylic acid (S18)

A mixture of 2-(4-fluorophenyl)-7-methyl-3\(^H\)-imidazo[4,5-b]pyridine (0.130 g, 0.572 mmol) and selenium dioxide (0.248 g, 2.231 mmol) in pyridine (15 ml) was heated at 110°C for 3 h. The hot reaction mixture was filtered through diatomaceous earth, washed with hot pyridine (10 ml) and methanol (20 ml), and the filtrate was concentrated and dried. The crude residue was dissolved in methanol (20 ml) followed by the dropwise addition of thionyl chloride (0.418 ml, 5.72 mmol) at room temperature. The reaction mixture was stirred at reflux for 5 h, cooled, concentrated,
suspended in ice-water (10 ml) and filtered. The pH of the filtrate was adjusted to 7 using conc. NH₄OH followed by extraction with DCM-iPrOH 4-1 (3 x 20 ml). The combined organic layers were washed with brine, dried over Na₂SO₄ and concentrated to provide the desired product as brown-yellow solid (0.13 g, 68% yield) A mixture of methyl 2-(4-fluorophenyl)-3H-imidazo[4,5-b]pyridine-7-carboxylate (0.130 g, 0.479 mmol) and lithium hydroxide (0.023 g, 0.959 mmol) was suspended in THF-water 1-1 (2 ml) and the reaction mixture was heated at 60°C for 18 h. The volatiles were evaporated, the residue redissolved in water and the pH adjusted to 3-4. The precipitate was filtered and washed with water to give white crystals.

1H NMR (400 MHz, DMSO-d₆) δ 12.93 - 12.72 (s, 1H), 8.54 - 8.48 (d, J = 5.0 Hz, 1H), 8.47 - 8.38 (dd, J = 8.8, 5.0 Hz, 2H), 7.65 - 7.62 (d, J = 5.0 Hz, 1H), 7.46 - 7.39 (t, J = 8.8 Hz, 2H); UPLC I (ESI) Rᵣ 1.19 min, m/z 258.5 [M+H]+ (96%); (0.1 g, 81%)

2-(4-methoxyphenyl)-7-methyl-3H-imidazo[4,5-b]pyridine (S19)
Was prepared in a similar manner as compound S17
1H NMR (400 MHz, DMSO-d₆) δ 8.27 – 8.08 (m, 3H), 7.50-7.45 (m, 1H), 7.12 (d, J = 8.2 Hz, 2H), 3.85 (s, J = 12.2 Hz, 3H), 2.59 (s, J = 8.4 Hz, 3H); UPLC I (ESI) Rᵣ 1.20 min, m/z 240.4 [M+H]+ (91%); (0.203, 32%)

2-(4-methoxyphenyl)-3H-imidazo[4,5-b]pyridine-7-carboxylic acid (S20)
Was prepared in a similar manner as compound S18
1H NMR (400 MHz, DMSO-d₆) δ 14.07 – 13.65 (m, 1H), 12.69 (s, 1H), 8.46 (s, 1H), 8.33 (d, J = 8.8 Hz, 2H), 7.59 (d, J = 5.0 Hz, 1H), 7.13 (d, J = 8.9 Hz, 2H), 3.86 (s, 3H); UPLC I (ESI) Rᵣ 1.17 min, m/z 270.5 [M+H]+ (99%);

Scheme S3

4-Bromo-1,7-naphthyridine (S21), 1,7-naphthyridine-4-carbonitrile (S22) and 1,7-naphthyridine-4-carboxylic acid (S23).

4-bromo-1,7-naphthyridine (S21)
A solution of 1,7-naphthyridin-4(1H)-one (0.552 g, 3.78 mmol) in DMF (5 ml) was treated with tribromophosphine (0.461 ml, 4.91 mmol) After 2 hours the mixture was added to water and
neutralized with saturated aqueous sodium bicarbonate solution. Filtration and drying afforded a yellow solid (0.5 g, 63%).

$^1$H NMR (400 MHz, DMSO-$d_6$) δ 9.45 (d, J = 0.89 Hz, 1H), 8.91 (d, J = 4.64 Hz, 1H), 8.78 (d, J = 5.77 Hz, 1H), 8.23 (d, J = 4.63 Hz, 1H), 8.00 (dd, J = 0.89, 5.78 Hz, 1H); UPLC (ESI) I R, 1.30, m/z 209.4, 211.4 [M+H]$^+$ (94%);

1,7-naphthyridine-4-carbonitrile (S22)
The cyanation procedure as described in Yu et al was used.$^5$ To a round bottom flask was added 4-bromo-1,7-naphthyridine (0.3 g, 1.435 mmol), zinc cyanide (0.101 g, 0.861 mmol), 10 wt % palladium on carbon (0.153 g, 0.144 mmol), 1,1'-Bis(diphenylphosphino)ferrocene (0.032 g, 0.057 mmol) and DMAC (2 ml). The resulting slurry was sparged with sub-surface argon for 10 min, and zinc(II) formate dihydrate (0.041 g, 0.215 mmol) was added to the reaction mixture. The reaction mixture was again sparged with sub-surface argon for 10 min and was heated under argon to 100-110°C for 3 h. The reaction mixture was diluted with 10 ml of EtOAc. The resulting slurry was filtered and the cake was rinsed with EtOAc (2 ml). The product was isolated by washing the filtrate with water (2 × 10 ml) and 5% NH$_4$OH (1 × 10 ml). The organic layer was dried with Na$_2$SO$_4$. The volatiles were removed in vacuo to give a residue, which was further purified by silica gel chromatography (EtOAc/heptane) to provide the product. (0.16 g, 72%)

$^1$H NMR (400 MHz, CDCl$_3$) δ 9.63 (s, 1H), 9.17 (d, J = 4.30 Hz, 1H), 8.83 (d, J = 5.75 Hz, 1H), 7.99 (dd, J = 0.97, 5.71 Hz, 1H), 7.94 (d, J = 4.31 Hz, 1H); 13C NMR (101 MHz, CDCl$_3$) δ 154.97, 151.13, 146.15, 142.88, 129.02, 128.66, 118.17, 117.00, 114.35; UPLC (ESI) I R, 1.09, m/z 156.5 [M+H]$^+$ (94%);

1,7-naphthyridine-4-carboxylic acid (S23)
A mixture of 1,7-naphthyridine-4-carbonitrile (0.09 g, 0.580 mmol), NaOH (0.048 g, 1.19 mmol) 1 ml of ethanol and 1 ml of water was heated to reflux for five hours. The reaction mixture was cooled to room temperature, and concentrated under reduced pressure. 2 M hydrochloric acid was added so that pH of the resultant residue became about 3, which was concentrated under reduced pressure again. UPLC (ESI) I R, 0.31, m/z 175.8 [M+H]$^+$ (94%); (0.095 g, 94%)

7-phenylquinoline-4-carboxylic acid (S24)
A mixture of methyl 7-bromoquinoline-4-carboxylate$^6$ (0.15 g, 0.564 mmol), phenylboronic acid (0.103 g, 0.846 mmol), tetrakis(triphenylphosphine)palladium(0) (0.033 g, 0.028 mmol), K$_3$PO$_4$ (0.598 g, 2.82 mmol), in 2 ml H$_2$O and 2 ml dioxane was stirred at 60°C for 1.5 h. EtOAc and water were
added then the organics were extracted with H₂O. The water was separated, acidified and the white precipitate was filtered to give 7-phenylquinoline-4-carboxylic acid (0.110 g, 0.441 mmol, 78 % yield)

\[ ^1H \text{NMR (400 MHz, DMSO-}d_6 \text{)} \delta 9.07 (d, J = 4.36 Hz, 1H), 8.81 (d, J = 8.91 Hz, 1H), 8.37 (d, J = 1.80 Hz, 1H), 8.09 (dd, J = 2.01, 8.94 Hz, 1H), 7.93 (d, J = 4.36 Hz, 1H), 7.92 - 7.88 (m, 2H), 7.67 - 7.59 (m, 1H), 7.60 - 7.51 (m, 2H); UPLC (ESI) tR 1.34, m/z 250.5 [M+H]^+ (94%)\]

**7-(phenylamino)quinoline-4-carboxylic acid (S25)**

A mixture of methyl 7-(phenylamino)quinoline-4-carboxylate (0.150 g, 0.539 mmol), NaOH (0.043 g, 1.078 mmol) in MeOH/H₂O (5 ml) (1/1) was stirred for 16 h at rt. the volatiles were evaporated, water was added and acidified to pH 2. This yielded a red precipitate, which was filtered to give 7-(phenylamino)quinoline-4-carboxylic acid (0.120 g, 84 % yield)

\[ ^1H \text{NMR (400 MHz, DMSO-}d_6 \text{)} \delta 8.82 - 8.79 (m, 2H), 8.54 (d, J = 9.25 Hz, 1H), 7.57 (t, J = 3.38 Hz, 2H), 7.43 (dd, J = 2.49, 9.27 Hz, 1H), 7.39 - 7.33 (m, 2H), 7.31 - 7.26 (m, 2H), 7.00 (tt, J = 1.23, 7.21 Hz, 1H); UPLC (ESI) R\text{t} 1.12 min, m/z 265.6 [M+H]^+ (96%).\]

I.8 4-methylisatin (S26), 6-methylisatin (S27), 5-methylquinoline-2,4-dicarboxylic acid (S28), 7-methylquinoline-2,4-dicarboxylic acid (S29), 5-methylquinoline-4-carboxylic acid (S30) and 7-methylquinoline-4-carboxylic acid (S31).

**Scheme S4**

![Scheme S4](image)

- a) MsOH, 55°C; b) NaOH, sodium pyruvate, H₂O, reflux; c) H₂O, pressure tube, 210°C

4-methylisatin (S26) and 6-methylisatin (S27)

The intermediate isonitrosoacetanilide was added in small portions, with stirring, to a 100 ml Erlenmeyer flask containing 5.15 ml concentrated methanesulfonic acid (1.06 ml, 16.3 mmol), which had been heated to 55°C. The temperature of the reaction mixture was maintained below 70°C during this addition. After all the isonitroso had been added, the dark-colored solution was heated at
80°C for an additional 10 min and then cooled to room temperature, poured onto 25 ml of crushed ice, and allowed to stand for 30 min. The precipitate was collected by filtration, washing three times with water, and dried under vacuum to yield a mixture of 4- and 6-methylisatin that was of sufficient purity to be used directly in the next step. (6-methylisatin, 40%, 1.02 g, 4-methylisatin 30%, 0.77 g)

\[^1^H\text{NMR}\ (400\text{MHz, DMSO-d}_6) \delta 10.98\ (s, 1H), 7.37\ (d, J = 7.7\text{ Hz, 1H}), 6.88 – 6.82\ (t, J = 7.7\text{ Hz, 1H}), 6.69\ (d, J = 7.8\text{ Hz, 1H}), 2.34\ (d, J = 2.4\text{ Hz, 3H}).\ UPLC I (ESI) R_t 1.30\text{ min, m/z 162.5}\ [\text{M+H}]^+

6-methylisatin

\[^1^H\text{NMR}\ (400\text{MHz, DMSO-d}_6) \delta 10.98\ (s, 1H), 7.42\ (t, J = 7.8\text{ Hz, 1H}), 6.88 – 6.82\ (m, 1H), 6.70\ (d, J = 2.5\text{ Hz, 1H}), 2.42\ (s, 3H).\ UPLC I (ESI) R_t 1.32\text{ min, m/z 162.5}\ [\text{M+H}]^+

5-methylquinoline-2,4-dicarboxylic acid (S28) and 7-methylquinoline-2,4-dicarboxylic acid (S29)

An aqueous solution of NaOH (72.6 ml, 17.57 g) was added to 6-methylindoline-2,3-dione (5.9 g, 36.6 mmol), and sodium 2-oxopropanoate (4.83 g, 43.9 mmol) was added. The mixture was refluxed for 4 h, cooled to rt and the products were precipitated by adjusting the pH to 2 with 2N HCl. The precipitate was filtered and purified using a Biotage KP-C18 flash column with a water-acetonitrile gradient with 0.1% formic acid as an additive.

5-methylquinoline-2,4-dicarboxylic acid: \[^1^H\text{NMR}\ (400\text{MHz, DMSO-d}_6) \delta 10.98\ (s, 1H), 8.00\ (s, 1H), 7.81\ (dd, J = 8.4, 7.1\text{ Hz, 1H}), 7.64\ (d, J = 7.0\text{ Hz, 1H}), 2.69\ (s, 3H);\ UPLC I (ESI) R_t 0.35\text{ min, m/z 232.5}\ [\text{M+H}]^+

7-methylquinoline-2,4-dicarboxylic acid: \[^1^H\text{NMR}\ (400\text{MHz, DMSO-d}_6) \delta 10.98\ (s, 1H), 8.39\ (s, 1H), 8.01\ (s, J = 6.8\text{ Hz, 1H}), 7.66\ (dd, J = 8.8, 1.9\text{ Hz, 1H}), 2.55\ (s, 3H);\ UPLC I (ESI) R_t 0.76\text{ min, m/z 232.5}\ [\text{M+H}]^+

5-methylquinoline-4-carboxylic acid (S30)

5-methylquinoline-2,4-dicarboxylic acid (0.8 g, 3.46 mmol) was dispersed in water and heated in a sealed pressure tube for 50 min at 208°C with stirring. After cooling the precipitate was collected and used without further purification.

\[^1^H\text{NMR}\ (400\text{MHz, DMSO-d}_6) \delta 8.89\ (d, J = 4.3\text{ Hz, 1H}), 7.93\ (d, J = 8.4\text{ Hz, 1H}), 7.68\ (dd, J = 8.4, 7.1\text{ Hz, 1H}), 7.49 – 7.44\ (m, 2H), 2.65\ (s, 3H);\ UPLC I (ESI) R_t 0.24\text{ min, m/z 188.5}\ [\text{M+H}]^+;\ (0.4g, 61%)

7-methylquinoline-4-carboxylic acid (S31)

Was prepared in a similar manner as 5-methylquinoline-4-carboxylic acid, but stirred for 90 min at 208°C in a sealed pressure tube.
1H NMR (400 MHz, DMSO-d$_6$) δ 8.98 (d, $J$ = 4.4 Hz, 1H), 8.62 – 8.57 (m, 1H), 7.92 – 7.89 (m, 1H), 7.85 (d, $J$ = 4.4 Hz, 1H), 7.56 (dt, $J$ = 8.5, 3.0 Hz, 1H), 2.54 (dd, $J$ = 6.0, 1.0 Hz, 3H); UPLC I (ESI) R$_t$ 0.34 min, m/z 188.5 [M+H]$^+$(0.6 g, 90%)

Section 1.9 5-Phenylquinoline-4-carboxylic acid (S32) and 5-methoxyquinoline-4-carboxylic acid (S33).

5-phenylquinoline-4-carboxylic acid (S32)

A mixture of 5-bromoquinoline-4-carboxylic acid (0.11 g, 0.436 mmol), phenylboronic acid (0.080 g, 0.655 mmol), Tetrakis(triphenylphosphine)palladium(0) (0.025 g, 0.022 mmol) and K$_3$PO$_4$ (0.463 g, 2.182 mmol) in 2 ml H$_2$O and 2 ml dioxane was stirred at 60°C for 1.5 h. EtOAc was added then the organics were washed with H$_2$O and brine then concentrated. The residue was suspended in cold EtOAc and the solids were collected by suction filtration, washed with cold EtOAc and dried to give 5-phenylquinoline-4-carboxylic acid (0.092 g, 0.369 mmol, 85% yield) as a solid.

1H NMR (400 MHz, CDCl$_3$) δ 9.13 (t, $J$ = 5.52 Hz, 1H), 8.35 (dd, $J$ = 20.9, 8.6 Hz, 1H), 8.15 (s, 1H), 7.94 (t, $J$ = 7.8 Hz, 1H), 7.87 (dd, $J$ = 11.1, 6.4 Hz, 1H), 7.77 (t, $J$ = 7.7 Hz, 1H), 7.68 (dt, $J$ = 4.5, 2.3 Hz, 1H), 7.54 - 7.41 (m, 2H), 7.19 (t, $J$ = 7.57 Hz, 1H); UPLC I (ESI) R$_t$ 1.11 min, m/z 250.5 [M+H]$^+$(96%).

5-methoxyquinoline-4-carboxylic acid (S33)

A mixture of 5-bromoquinoline-4-carboxylic acid (0.1 g, 0.397 mmol), sodium methoxide (0.744 ml, 3.97 mmol), DMF (2 ml) and MeOH (5 ml) was stirred and heated at 90°C under nitrogen. copperbromide (0.028 g, 0.198 mmol) was added and the reaction mixture was maintained at 90°C for 16 h, poured into water and washed with ether. The water layer was acidified, and extracted with ether (3x). The organic layers were washed with water and brine, dried (MgSO$_4$), filtered and the filtrate concentrated. (0.07 g, 87%)

1H NMR (400 MHz, CDCl$_3$) δ 8.64 (d, $J$ = 5.8 Hz, 1H), 7.65 - 7.60 (m, 1H), 7.57 (dd, $J$ = 8.4, 1.3 Hz, 1H), 6.92 (dd, $J$ = 7.8, 1.2 Hz, 1H), 6.87 (d, $J$ = 5.8 Hz, 1H), 3.96 (s, 3H); UPLC I (ESI) R$_t$ 1.09 min, m/z 204.5 [M+H]$^+$(96%)

Section 1.10 2-Amino-1-(pyrrolidin-1-yl)ethanone hydrochloride (S34) and (S)-tert-butyl 2-(2-chloroacetyl)pyrrolidine-1-carboxylate (S35).

2-amino-1-(pyrrolidin-1-yl)ethanone hydrochloride (S34)
4 N HCl in dioxane (3.29 ml, 13.14 mmol) was added to tert-butyl 2-oxo-2-(pyrrolidin-1-yl)ethylcarbamate (0.3 g, 1.314 mmol), the mixture was stirred for 16 h and evaporated, the residue was washed with ether to give white crystals. $^1$H NMR (400 MHz, MeOD) $\delta$ 3.66 (s, 2H), 3.47 (m, 4H), 2.06 - 1.97 (m, 2H), 1.96 - 1.88 (m, 2H); UPLC I (ESI) R$_t$ 0.29 min, m/z 129.5 [M+H]$^+$ (95%);(0.186 g, 86%)

(S)-tert-Butyl 2-(2-chloroacetyl)pyrrolidine-1-carboxylate (S35) $^{23}$

A solution of lithium diisopropylamide (15.20 ml, 27.4 mmol) was added dropwise over 30 min to a solution of (S)-1-tert-butyl 2-methyl pyrrolidine-1,2-dicarboxylate (1.4 ml, 6.84 mmol) and methylene chloroiodide (2.0 ml, 27.4 mmol) in THF (5.5 ml) at -78°C. The internal temperature of the reaction mixture was kept below -70°C during the addition and the reaction mixture was stirred for 10 min at -75°C. A solution of acetic acid (1.5 ml) in THF (10 ml) was added dropwise over 10 min, keeping the internal temperature below -65°C. After stirring an additional 10 min at -75°C, the reaction mixture was partitioned between ethyl acetate (75 ml) and brine (75 ml). The organic layer was washed with saturated NaHCO$_3$ solution (2 x 50 ml), 5% NaHSO$_3$ solution (2 x 50 ml) and brine (50 ml), dried (MgSO$_4$) and concentrated affording a dark yellow solid. The residue was chromatographed on 2.5 x 12 cm silica gel using 7 to 35% ethyl acetate/hexane affording 1.2 g, 70% of chloromethylketone as a yellow oil. $^1$H NMR (400 MHz, CDCl$_3$) (cis/trans 1-1 mixture of carbamate rotamers) $\delta$ 4.52 (dd, J = 4.23, 8.52 Hz, 0.5H), 4.46 (dd, J = 4.52, 8.46 Hz, 0.5H), 4.36 - 4.26 (m, 1H), 4.22 (d, J = 3.52 Hz, 1 H), 3.59 - 3.38 (m, 2H), 2.32 - 2.11 (m, 1H), 1.92 (m, 3H), 1.45 (s, 4.5H), 1.41 (s, 4.5H); UPLC I (ESI) R$_t$ 1.74 min, m/z 192.5 [M+H-tert-Bu]$^+$ (98%)

II. Enzymatic assay data

II.1 Enzyme purification and assay conditions for IC$_{50}$ determinations

Enzyme purification

- Recombinant murine FAP was purified from the cultured supernatant of HEK293 human embryonic kidney cell line as described elsewhere (Cheng, J. et al. Cancer Res. 2002, 62, 4767-4722).

- Recombinant human PREP was expressed in and purified from E coli as previously described (Szeltner, Z.; Renner, V.; Polgar, L. Prot. Sci. 2000, 9, 353-360).

- DPP IV and DPPII were purified from human seminal plasma as described previously. (De Meester, I.; Vanhoof, G.; Lambeir, A.; Scharpe, S. J. Immun. Methods 1996, 189, 99-105 and Maes, M. B.; Lambeir,
A. M.; Gilany, K.; Senten, K; Van der Veken, P; Leiting, B.; Augustyns, K.; Scharpe, S; De Meester, I. Kinetic investigation of human dipeptidyl peptidase II (DPPII)-mediated hydrolysis of dipeptide derivatives and its identification as quiescent cell proline dipeptidase (QPP)/dipeptidyl peptidase 7 (DPP 7). *Biochem. J.* 2005, 386, 315-324

Recombinant human DPP8 was expressed and purified as described. (Chen, Y. S.; Chien, C. H.; Goparaju, C. M.; Hsu, J. T.; Liang, P. H.; Chen, X. Purification and characterization of human prolyl dipeptidase DPP8 in SF9 insect cells. *Prot. Exp. Purif.* 2004, 35, 142-146)

DPP9 was purified from bovine testes as described by Dubois et al. (Dubois, V.; Lambeir, A. M.; Van der Veken, P.; Augustyns, K.; Creemers, J.; Chen, X.; Scharpe, S.; De Meester, I. Purification and characterization of dipeptidyl peptidase IV-like enzymes from bovine testes. *Front. Biosci.* 2008, 13, 3558–3568)

**IC₅₀-determination for FAP and PREP**

Enzyme activities were determined kinetically in a final volume of 200 µl for 10 minutes at 37°C by measuring the initial velocities of pNA release (405 nm) from the substrate using a Spectramax plus microtiterplate reader (Molecular devices). One unit of enzyme activity was defined as the amount of enzyme that catalyzes the release of 1 µmol pNA from the substrate per minute under assay conditions. All measurements were carried out in duplicate. The IC₅₀ value was defined as the inhibitor concentration which caused a 50% decrease of the activity under assay conditions.

The chromogenic substrate Ala-Pro-p-nitroanilide (2 mmol/l) was used at pH 7.4 for FAP activity measurement. The substrate concentrations were chosen around the Km value obtained under the assay conditions used. Buffer compositions for the DPP assays were previously reported in the purification articles– vide supra. The FAP assay buffer consisted of 50 mM Tris pH7.4 containing 100 mmol/l NaCl and 0.1 mg/ml bovine serum albumin. The PREP activity was measured as described by Brandt et al. using the chromogenic substrate Z-Gly-Pro-p-nitroanilide (0.25 mmol/l) at pH 7.5 in the presence of 10 mmol/l DTT. (Brandt, I.; Gérard, M.; Sergeant, K.; Devreese, B.; Baekelandt, V.; Augustyns, K.; Scharpé, S.; Engelborghs, Y.; Lambeir, A.M. *Peptides* 2005, 26, 2536-2546)

**IC₅₀-determination for DPPII, DPPIV, and DPP 9**

Initial rates were determined kinetically in a final volume of 200 µl for 10 minutes at 37°C by measuring the initial velocities of pNA release (405 nm) from the substrate using a Spectrafluor Plus reader (Tecan Benelux). The chromogenic substrate Gly-Pro-p-nitroanilide (100 µmol/l) was used at
pH 8.3 for DPP IV, Lys-Ala-p-nitroanilide (1 mmol/l) at pH 5.5 for DPP II and Ala-Pro-p-nitroanilide
(300 µmol/l) at pH 7.4 for DPP9-activity measurement. The substrate concentrations were chosen around the Km value obtained under the assay conditions used. Buffer compositions were reported before. (Dubois, V.; Lambeir, A. M.; Van der Veken, P.; Augustyns, K.; Creemers, J.; Chen, X.; Scharpe, S.; De Meester, I. Purification and characterization of dipeptidyl peptidase IV-like enzymes from bovine testes. *Front. Biosci.* **2008**, *13*, 3558–3568)

Test compounds were dissolved and diluted in DMSO (final concentration of DMSO during assay was 5% v/v). Inhibitors were pre-incubated with the enzyme for 15 min at 37 °C before starting by the addition of substrate. The concentrations of enzyme and inhibitor during the preincubation were the double of the final concentrations during the initial rate measurement. All measurements were carried out in duplicate. The initial evaluation of compounds was carried out at 100 µmol/l, or in case of solubility limits, the highest concentration possible. If vi/vo (initial velocity in presence of inhibitor/velocity in presence of DMSO) was < 0.5, an IC$_{50}$ value was determined experimentally using at least 8 different concentrations of inhibitor. For those compounds with IC$_{50}$ values below 5 µmol/l for one of the enzymes, the analysis was repeated using a new stock of compound. Generally, independent measurements of IC$_{50}$ differed less than 20% from each other. The IC$_{50}$-value was defined as the inhibitor concentration, which caused a 50% decrease of the activity under assay conditions. The total inhibitor concentration is represented by I0. IC$_{50}$-values were calculated with the GraFit software (GraFit Version 5, Leatherbarrow, R.J., Erithacus Software Ltd., Horley, U.K.) using eq (1).

\[
\frac{v_i}{v_o} = \frac{1}{1 + \left(\frac{I0}{IC_{50}}\right)^s} + \text{background}
\]

eq. (1)

where s is the slope factor and background represents the estimated minimal vi/vo value. The errors given in the tables represent standard errors of the fit unless otherwise specified.

**II.2 Ex vivo Enzyme activity assays**

**Protocol for determination of FAP-activity in plasma of inhibitor-treated rats:**

FAP-activity in plasma was determined by means of an enzymatic end-point method using Z-Gly-Pro-AMC as a fluorogenic substrate. First, samples were diluted 101 times in a buffer containing 0.1 M
Tris buffer containing 400 mM sodium chloride, 50 mM salicylic acid, 1 mM sodium azide, 1 mM EDTA at pH 7.5. To 100 µl of diluted sample was added 10 µl of 4.6 mM Z-Gly-Pro-AMC in acetonitrile:water (40:60) and incubated during 15 minutes at 37°C. Sample and substrate blank tubes were also incubated. The enzymatic reaction was stopped by adding 0.5 ml of 1.5 M acetic acid. The fluorescence of the liberated AMC was measured as described above. Within and between-run CV was 1.9 and 3.9% respectively. Specificity of the method was assessed by use of specific FAP-inhibitors. Addition of specific PREP-inhibitors demonstrated the absence of interference by active PREP in this assay that did not contain DTT (PREP in plasma requires treatment with DTT in order to be active). The influence of dilution on the read-out of the protocol was determined as described in:


III. In vivo PK experiments

III.1.1 Animals

Male Wistar rats (BW~250 g; Janvier, France) were housed individually and randomly allocated to 9 groups of 3 animals each, based on body weight. One of these 9 groups served as a control (that was not treated with inhibitor). The animals were fasted overnight until 4 hours after dosing. Drinking water remained available ad libitum throughout the experiment.

III.1.2 Inhibitor formulation

The inhibitors were dissolved in PEG200 at 10 mg/mL. The same formulation was used both for oral and parenteral dosing. The test formulation was freshly prepared and kept at room temperature before administration.

III.1.3 Dosing and blood sampling frequency
Six male rats were treated for each inhibitor tested, three of which (one group, vide supra) received the compound via a single intravenous (IV, tail vein) administration at 5 mg/kg. The other three animals were dosed per os (PO) at 20 mg/kg. Blood samples were collected at 0.083 h, 0.25 h, 0.5 h, 1 h, 2 h, 4 h, 6h and 24 h post administration.

III.1.4 Blood and tissue sampling procedure and sample processing

Blood (300 µL) was collected on ice in Serum-Gel Clotting Activator 1.1 mL microtubes (Sarstedt) upon intracardial puncture. After centrifugation (10,000 g, 5 min), the collected serum was divided into 2 aliquots and stored at -80°C. Sample processing consisted of adding 4 parts of cold acetonitrile to 1 part of serum; the mixture was vortexed for 30 sec and centrifuged at 4°C for 5 minutes at 15,000 rpm. The supernatant was further diluted (1/20) in solvent (90/10 H₂O/ACN). At each sampling time point, 1 aliquot of serum was stored at -80°C for FAP activity measurements. The overall dilution factor of the samples was 100.

III.1.5 Determination of plasma levels using UPLC-MS.

The analysis of the serum was performed using a Waters Acquity H-class UPLC system coupled to a Waters TQD ESI mass spectrometer and a Waters TUV detector. A Waters Acquity UPLC BEH C18 1.7µm 2.1 x 50 mm column was used. The samples were analyzed in triplicate. A standard curve of six points was made to determine the measured concentrations.

The following parent and daughter peaks were used:

<table>
<thead>
<tr>
<th>Compound Number</th>
<th>Parent ion</th>
<th>Daughter peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>309.14</td>
<td>185.11</td>
</tr>
<tr>
<td>5</td>
<td>389.104</td>
<td>171.15</td>
</tr>
<tr>
<td>60</td>
<td>345.14</td>
<td>185.08</td>
</tr>
<tr>
<td>61</td>
<td>375.13</td>
<td>159.06</td>
</tr>
</tbody>
</table>

Standard pharmacokinetic parameters were determined using the Topfit™ software: peak time (Tmax), peak concentration (Cmax), elimination half-life (T1/2), systemic availability (AUC0-t), volume of distribution (Vz) and clearance (Cl).
IV. In vitro PK experiments

Kinetic solubility protocol:
A turbidimetric method was used. First a series of DMSO compound stock solutions was prepared (0.15-5 mM) An aliquot of 4µL stock solution was added to 196µL PBS buffer (pH 7.4). A series of concentrations were prepared (3.13-200 µM), including a blank on a microtiter plate. (2% DMSO – D6) The microtiter plate was shaken for 10 seconds and incubated for 2 hours at 37°C. When there was no turbidity measured at a given concentration, the sample was assumed to be dissolved.

Log D protocol
20 µL of a 10 mM DMSO stock solution of the compound was added to 990 µL saturated PBS (pH 7.4). To this mixture was 990 µL of octanol added. The mixture was shaken for 2 hours at 37°C, then at room temperature for 10 minutes. 4 µL of octanol was added to 996 µL methanol and analyzed in triplicate. The PBS is injected directly without any further dilution and analyzed in triplicate. The experiment was done in duplicate. A standard curve of six points was made to fit the measured concentrations.

Plasma stability protocol
5 µl of a 10mM stock solution of the compound in DMSO was added to 995 µl of plasma to obtain a 50 µM final solution. The mixture was gently shaken for 6 h at 37°C. Aliquots of 100 µl were taken at various time points (0, 0.5h, 1 h, 2 h, 3 h, 6 h) and diluted with 400 µl of cold methanol (stored at 4°C). The suspension was centrifuged at 14000 rpm for 5 min. 50 µl of the supernatant was diluted with 950 µl of methanol and analyzed with LC/MS/MS. The samples were analyzed in triplicate. The experiment was done in duplicate. A standard curve of six points was made to fit the measured concentrations. Enalapril maleate was used as a control in mice, rats, and in human.

Metabolic stability protocol
Liver microsomes (20 mg protein/ml), NADPH regenerating system solutions A & B and 5 mM stock compound solution (100% DMSO) are prepared. The reaction mixture finally contains 713 µL purified water, 200 µL 0.5 M potassium phosphate pH 7.4, 50 µL NADPH regenerating system solution A (BD Biosciences Cat. No. 451220), 10 µL NADPH regenerating system solution B (BD Biosciences Cat. No. 451200) and 2 µL of the compound stock solution (10 µM final concentration). The reaction mixture is warmed to 37°C for 5 minutes in a water bath and the reaction is initiated by addition of 25 µL of liver microsomes (0.5 mg protein/ml final concentration). At different time points (0 min – 15 min –
30 min – 60 min – 120 min – 240 min – 360 min – 24h), 20 µL is withdrawn and 80 µL cold acetonitrile is added on ice for 10 minutes. Then the mixtures are centrifuged at 13 000 rpm for 5 min at 4°C. The supernatant is further diluted in 90% water/MeCN (1/25) to fall within the range of the LC/MS/MS analysis. At each time point, the compound is analyzed using LC/MS/MS with the same UPLC system as described above. A standard curve of six points was made to fit the measured concentrations. Verapamil was used as a positive control.

**PBS buffer stability protocol**

A µM solution of the compound in DMSO was prepared. 5 µL of this solution was added to a PBS buffer (pH 7.4) to give a final concentration of 1 µM. The mixture was stirred for 24h at 37°C. At different time points (0 min – 15 min – 30 min – 60 min – 120 min – 240 min – 360 min – 24h), 20 µL is withdrawn, diluted to 100 µL and analyzed using LC/MS/MS with the same UPLC system as described for the *in vivo* pK experiments. A standard curve of six points was made to fit the measured concentrations.

**Detailed Cytotoxicity data**

**Assay protocol:**

**Cell culture:**

MRC-5 cells (human embryonic diploid fibroblasts) were obtained from Sigma and cultured in MEM + Earl’s salts-medium, supplemented with l-glutamine, NaHCO₃ and 5% inactivated fetal calf serum. All cultures and assays were conducted at 37°C under an atmosphere of 5% CO₂. All media, solvents, supplements, plates and reagents used in these experiments were also obtained from Sigma.

**Compound solutions/dilutions:**

Compound stock solutions were prepared in 100% DMSO at 20 mM. The compounds were serially pre-diluted (2-fold or 4-fold) in DMSO followed by a further (intermediate) dilution in demineralized water to assure a final in-test DMSO concentration of <1%.

**Compound assays:**
The compounds were tested at 5 concentrations (64 - 16 - 4 - 1 and 0.25 \( \mu \text{M} \)). Assays were performed in sterile 96-well microtiter plates, each well containing 10 \( \mu \text{l} \) of the aqueous compound dilutions together with 190 \( \mu \text{l} \) of MRC-5 SV2 inoculum (3x10^4 cells/ml). Cell growth was compared to untreated control wells (100% cell growth) and medium control wells (0% cell growth). After 3 days of incubation, cell viability was assessed fluorimetrically after addition of 50 \( \mu \text{L} \) resazurin solution (50 \( \mu \text{g/mL} \) in PBS-buffer) per well. After 4 hours at 37°C, fluorescence was measured (\( \lambda_{\text{ex}} 550 \text{ nm}, \lambda_{\text{em}} 590 \text{ nm} \)). The results were obtained as % reduction in cell growth/viability compared to control wells, based on which an IC\(_{50}\) value was determined. A compound was classified as non-toxic when the IC\(_{50}\) value was higher than 30 \( \mu \text{M} \). Between 10 and 30 and \( \mu \text{M} \), the compound was regarded as moderately toxic. When the IC\(_{50}\)-value was lower than 10 \( \mu \text{M} \), the compound is classified as highly toxic. Cytotoxic reference compounds included were vinblastine and paclitaxel (IC\(_{50}\) <0.01 \( \mu \text{M} \)).

<table>
<thead>
<tr>
<th>Compound Nr</th>
<th>MRC-5, IC(_{50}) (( \mu \text{M} ))</th>
<th>Activity score MRC-5</th>
<th>Follow-up</th>
</tr>
</thead>
<tbody>
<tr>
<td>UAMC-01063-4</td>
<td>&gt; 64,00</td>
<td>&lt; 1</td>
<td>non-toxic</td>
</tr>
<tr>
<td>UAMC-01109-29</td>
<td>&gt; 64,00</td>
<td>&lt; 1</td>
<td>non-toxic</td>
</tr>
<tr>
<td>UAMC-01110-60</td>
<td>&gt; 64,00</td>
<td>&lt; 1</td>
<td>non-toxic</td>
</tr>
<tr>
<td>UAMC-01233-5</td>
<td>&gt; 64,00</td>
<td>&lt; 1</td>
<td>non-toxic</td>
</tr>
<tr>
<td>UAMC-01241-52</td>
<td>&gt; 64,00</td>
<td>&lt; 1</td>
<td>non-toxic</td>
</tr>
<tr>
<td>UAMC-01285-61</td>
<td>&gt; 64,00</td>
<td>&lt; 1</td>
<td>non-toxic</td>
</tr>
<tr>
<td>UAMC-01532-66</td>
<td>&gt; 64,00</td>
<td>&lt; 1</td>
<td>non-toxic</td>
</tr>
<tr>
<td>UAMC-01530 57</td>
<td>28,84</td>
<td>= 2</td>
<td>slightly toxic</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Screening panel</th>
<th>Date</th>
<th>Reference validation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 MRC-5</td>
<td>11/01/2013</td>
<td>Tamoxifen 10,73</td>
</tr>
</tbody>
</table>
V. References


