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Synthesis and Evaluation of Indole Aspartyl Ketones as Novel Caspase-3 Inhibitors

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Synthesis, biological evaluation and structure-activity relationships for a series of novel nonpeptide small molecule inhibitors of caspase-3 are described. Among the synthesized compounds, 2,3,5,6-tetrafluorophenoxymethyl ketone derivatives of indole-N-acetamides have been identified as potent inhibitors of caspase-3. The most active compound within this series (**9a**) inhibited caspase-3 with an IC_{50} = 0.64 μM.

Key Words: Caspase inhibitors, Cysteine proteases, IDN-6556, Indole-N-acetic acid.

INTRODUCTION

The caspases[1] are a family of cysteine proteases with aspartic acid specificity at P1 involved in both cytokine maturation and apoptosis[2]. Based on its biological function, caspases can be divided into two groups. One of the groups, represented by caspase-1, activates interleukin-1 and plays an important function in cytokine maturation and inflammation[3]. The other group, including caspase-3, -8 and -9 plays a critical role in apoptosis by cleaving numerous important proteins[1a]. Because of the important function of caspases in both inflammation and apoptosis, the discovery and development of caspase inhibitors could result in novel antiinflammatory and anti-apoptotic drugs for the treatment of a variety of human diseases such as ischemia-reperfusion injury, cardiomyopathy, neurodegeneration, sepsis, type-1 diabetes, fulminant liver disease and allograft rejection[4,5].

Many caspase inhibitors have been designed and synthesized[6]. These include peptide based inhibitors[7], peptidomimetic based inhibitors[8], as well as non-peptide inhibitors such as isatins discovered through screening of compound libraries[9]. Some of these are selective for specific caspases, while others are broad-spectrum caspase inhibitors. IDN-6556 (IC_{50} = 0.06 μM) (Fig. 1) was identified by idun pharmaceuticals as a dipeptide based irreversible and broad-spectrum caspase inhibitor[10,11]. Herein, we have developed a series of novel non-peptidyl caspase inhibitors with significant activity. We also explored the replacement of 2,3,5,6-tetrafluorophenoxymethyl ketone by 2,6-difluorophenoxymethyl ketone as warhead, as this is very well explored in literature as peptide based caspase-3 inhibitors.

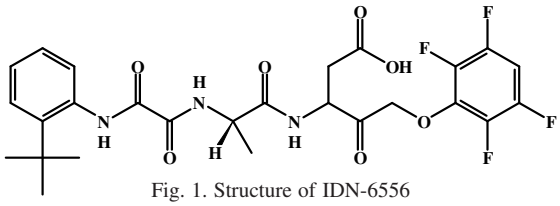
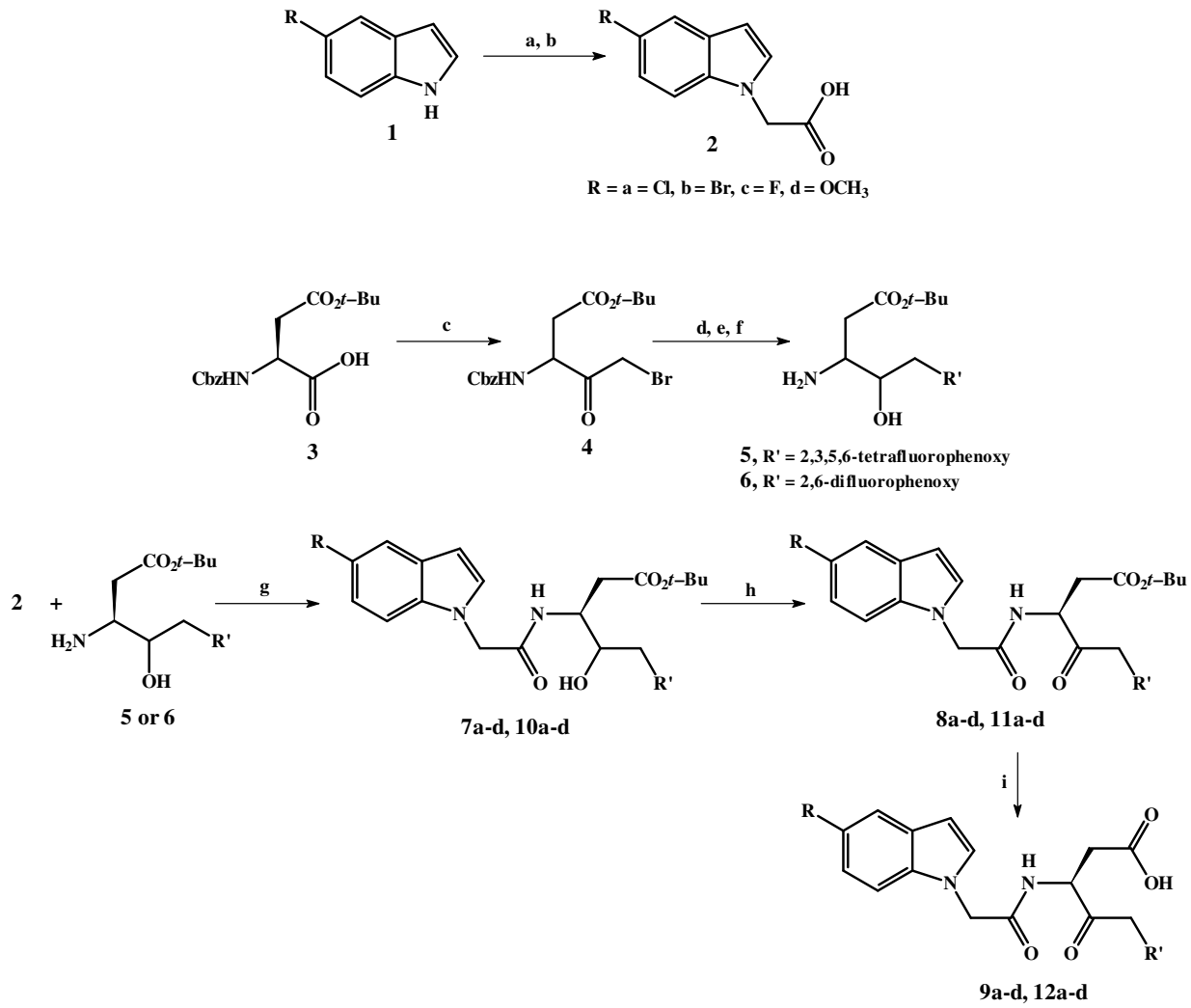


Fig. 1. Structure of IDN-6556

EXPERIMENTAL

NMR spectra were measured on a 300 or 400 MHz Varian Mercury plus instrument in DMSO-d_6 or CD_3OD as reference solvent and chemical shifts were expressed as δ. Coupling constants *J* are given in Hz. Tetramethylsilane (TMS) was used as an internal standard for ^{1}H NMR. Mass spectra (MS) were measured on a Single Quad Dual mode (APCI and ESI)-Agilent or Triple quad API 2000-Applied Biosystems. HPLC particulars: Agilent 1100 series having PDA detector at λ = 210 nm, flow 1.0 mL/min, column: Zorbax-C_{18}, pore size 5 μm, diameter × length = 4.6 × 150 mm; method:gradient elution with (A) as 5 mM ammonium acetate and (B) acetonitrile: methanol (1:1) for 12 min with 30-100-30 %. IR spectra (KBr) were recorded on a Perkin-Elmer spectrum one FT-IR spectrophotometer and band positions were reported in wave numbers (cm^{-1}). Column chromatography was performed using silica gel (60-120 mesh). Melting points were determined on a Stuart Scientific melting point apparatus and are uncorrected. All commercially available reagents were used as received.

The caspase-3 inhibitors ((**9a-d** and **12a-d**) were prepared as described in **Scheme-I**. In this study the intermediate (**2**) was prepared by alkylation of indole with ethyl 2-bromoacetate,



Scheme-I: Reagents and conditions: (a) ethyl 2-bromoacetate, K_2CO_3, DMF, rt, 2 h; (b) $LiOH·H_2O$, THF/H_2O (1:1), 0 ºC, 1 h; (c) (i) isobutyl chloroformate, NMM, THF, -10 ºC, 1 h; (ii) CH_2N_2/Et_2O, 0 ºC, 0.5 h; (iii) HBr (aq. 48 %), THF, 0 ºC, 1 h; (d) 2,3,5,6-tetrafluorophenol/2,6-difluorophenol, KF, DMF, rt, 1 h; (e) $NaBH_4$, EtOH, 0 ºC, 0.5 h; (f) H_2 (1 atm), 10 % Pd/C, MeOH, rt, 4 h; (g) EDCI, HOBt, DIEA, DMF, rt, 15 h; (h) Dess-Martin periodinane, CH_2Cl_2, 0 ºC, 2 h; (i) TFA, CH_2Cl_2, rt, 2 h

followed by basic hydrolysis to yield the indole-N-acetic acid[12]. The key intermediates (**5** and **6**) were prepared using commercially available Z-Asp (O-*t*Bu)-OH (**3**). Bromomethyl ketone (**4**) was obtained by treatment of the mixed anhydride with diazomethane[13] followed by displacement of the azide with hydrobromic acid. Phenoxy methyl ketones were synthesized by treating (**4**) under modified Finkel-stein[14] conditions with the appropriate phenol in DMF. Reduction of ketone and hydrogenolysis afforded the key intermediate (**5** or **6**). Coupling of acid (**2a-d**) with key intermediate (**5**) gave amide (**7a-d**), which was oxidized by Dess-Martin periodinane to produce the corresponding ketone (**8a-d**). Trifluoroacetic acid catalyzed cleavage of the *t*-butyl ester gave the free acid (**9a-d**). Similarly when acid (**2a-d**) was coupled with key intermediate (**6**) gave amide (**10a-d**), which was oxidized by Dess-Martin periodinane followed by *t*-butyl ester hydrolysis with trifluoroacetic acid gave the free acid (**12a-d**). Overall synthetic steps are easy to handle, synthetic intermediates are air stable.

General procedure for the synthesis of 7a-d and 10a-d: Acid (**2**) (108 mg, 0.5 mmol) was dissolved in anhydrous DMF (5 mL), diisopropylethylamine (0.26 mL, 1.5 mmol), 1-hydroxybenzotriazole (77 mg, 0.57 mmol) and amine (**5** or **6**) (201 mg, 0.57 mmol) were added and cooled to 0 ºC. 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (109 mg, 0.57 mmol) was then added and the reaction stirred at room temperature for 15 h. It was diluted with ice water and extracted with ethyl acetate (2 × 100 mL). The combined organic layers were washed with cold 0.5 N HCl (to remove excess of amine) followed by saturated aqueous sodium bicarbonate and saturated brine solution. The organic layer was dried over anhydrous sodium sulphate and concentrated in vacuum to afford (**7**) as pale yellow solid (62-100 %), which could be used for the next reaction without purification.

General procedure for the synthesis of 8a-d and 11a-d: To the slurry of Dess-Martin periodinane (270 mg, 0.63 mmol) in dry CH_2Cl_2 (10 mL) at 0 ºC was added a solution of **7** (290 mg, 0.53 mmol) in dry CH_2Cl_2 (5 mL) drop-wise over a period of 10 min and stirred at same temperature for 90 min. The reaction mixture was quenched with 1.0 g of sodium thiosulphate dissolved in 5 mL of 10 % sodium bicarbonate solution and stirred for 0.5 h and seperated organic layer and extracted aqueous layer with CH_2Cl_2 (2 × 25 mL). The combined

organic layers were washed with water followed by saturated brine solution and dried over anhydrous sodium sulphate and concentration was followed by purification by chromatography using silica gel (60-120 mesh) eluted with dichloromethane/methanol = 10:0.5 to afford **8** as cream colour solid (29-71 %).

General procedure for the synthesis of 9a-d and 12a-d: Trifluoroacetic acid (1.0 mL) was added to the cooled solution of **8** (160 mg, 0.3 mmol) in dry CH_2Cl_2 (5 mL) and anisole (0.2 mL). The reaction mixture was stirred at 0 ºC for 1-2 h. All volatiles were removed under reduced pressure and the crude purified by preparative TLC (dichloromethane/methanol = 10/1) to afford **7** as cream colour solid (6-68 %).

(*S*)-3-(2-(5-Chloro-1*H*-indol-1-yl)acetamido)-4-oxo-5-(2,3,5,6-tetrafluorophenoxy)pentanoic acid (9a): Yield: (0.085 g, 59 %); m.p. 188-191 ºC; IR (KBr, ν_{max}, cm^{-1}): 3318, 2950, 1746, 1704, 1662, 1517, 1489, 1290; ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 8.75 (d, *J* = 7.5 Hz, 1H, NH), 7.65-7.55 (m, 2H, Ar), 7.45-7.35 (m, 2H, Ar), 7.05 (dd, *J* = 8.7 Hz, *J* = 1.8 Hz, 1H, Ar), 6.42 (d, *J* = 3.2 Hz, 1H, Ar), 5.20 (d, *J* = 17.1 Hz, 1H, $COCH_2$), 5.05 (d, *J* = 17.1 Hz, 1H, $COCH_2$), 4.95 (d, *J* = 3 Hz, 2H, NCH_2CO), 4.50 (m, 1H, CHCO), 2.65-2.55 (dd, *J* = 16.8 Hz, *J* = 6.1 Hz, 1H, CH_2COOH), 2.55-2.40 (m, 1H, CH_2COOH); ^{13}C NMR (CD_3OD, 400 MHz) δ: 176.8, 176.2, 148.9, 146.6, 143.2, 140.7, 136.4, 131.7, 131.57, 131.36, 126.5, 122.8, 121, 111.6, 102.7, 102, 100.9 (m), 76.8, 54.1, 50.3, 38; MS: m/z = 485.2 (M^+-1); HPLC purity: 97 % (area %).

(*S*)-3-(2-(5-Bromo-1*H*-indol-1-yl)acetamido)-4-oxo-5-(2,3,5,6-tetrafluorophenoxy) pentanoic acid (9b): Yield: (0.009 g, 6 %); m.p. 199-201 ºC; IR (KBr, ν_{max}, cm^{-1}): 3318, 2950, 1745, 1704, 1662, 1517, 1489, 1290; ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 12.80-12.45 (brs, 1H, COOH), 8.80 (s, 1H, NH), 7.75 (d, *J* = 0.5 Hz, 1H, Ar), 7.70-7.55 (m, 1H, Ar), 7.40-7.30 (m, 2H, Ar), 7.25-7.15 (d, *J* = 8.1 Hz, 1H, Ar), 6.45 (d, *J* = 6.2 Hz, 1H, Ar), 5.40-5.15 (m, 2H, $COCH_2$), 4.95 (s, 2H, NCH_2CO), 4.70 (m, 1H, CHCO), 2.80-2.70 (m, 2H, CH_2COOH); MS: m/z = 531.0 (M^+-2); HPLC purity: 91 % (area %).

(*S*)-3-(2-(5-Fluoro-1*H*-indol-1-yl)acetamido)-4-oxo-5-(2,3,5,6-tetrafluorophenoxy) pentanoic acid (9c): Yield: (0.038 g, 43 %); m.p. 60-63 ºC; IR (KBr, ν_{max}, cm^{-1}): 3314, 2952, 1740, 1704, 1666, 1513, 1488, 1232; ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 8.75 (d, *J* = 9.1 Hz, 1H, NH), 7.65-7.55 (m, 1H, Ar), 7.45-7.25 (m, 3H, Ar), 6.95 (t, 1H, Ar), 6.45 (d, *J* = 6.3 Hz, 1H, Ar), 5.20-5.0 (m, 2H, $COCH_2$), 4.95 (s, 2H, NCH_2CO), 4.70 (m, 1H, CHCO), 2.80-2.70 (d, *J* = 9.2 Hz, 2H, CH_2COOH); ^{13}C NMR (CD_3OD, 400 MHz) δ: 174.6, 170.8, 160.57, 158.2, 148.9, 146.59, 143.2, 140.7, 138.6, 134.6, 131.9, 131.7, 130.6, 111.2 (m), 106.4, 106.2, 103, 101 (m), 76.2, 52.9, 50.4, 35.69; MS: m/z = 471.1 (M^+ + 1); HPLC purity: 97 % (area %).

(*S*)-3-(2-(5-Methoxy-1*H*-indol-1-yl)acetamido)-4-oxo-5-(2,3,5,6-tetrafluorophenoxy) pentanoic acid (9d): Yield: (0.058 g, 42 %); m.p. 58-62 ºC; IR (KBr, ν_{max}, cm^{-1}): 3305, 2950, 1740, 1704, 1661, 1518, 1490, 1243; ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 8.70 (d, *J* = 8.5 Hz, 1H, NH), 7.65-7.55 (m, 1H, Ar), 7.30-7.20 (m, 2H, Ar), 7.05 (d, *J* = 3.1 Hz, 1H, Ar), 6.70 (dd, *J* = 12.1 Hz, *J* = 3.2 Hz, 1H, Ar), 6.35 (d, *J* = 3.1 Hz, 1H, Ar), 5.30-5.05 (m, 2H, $COCH_2$), 4.85 (s, 2H, NCH_2CO), 4.70 (m, 1H, CHCO), 3.75 (s, 3H, OCH_3), 2.80-2.70 (m, 2H, CH_2COOH); MS: m/z = 483.3 (M^+ + 1); HPLC purity: 97 % (area %).

(*S*)-3-(2-(5-Chloro-1*H*-indol-1-yl)acetamido)-5-(2,6-difluorophenoxy)-4-oxopentanoicacid (12a): Yield: (0.09 g, 68 %); m.p. 169-172 ºC; IR (KBr, ν_{max}, cm^{-1}): 3307, 2945, 1736, 1658, 1594, 1499, 1475, 1292; ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 8.75 (d, *J* = 8.4 Hz, 1H, NH), 7.60 (s, 1H, Ar), 7.45-7.35 (m, 2H, Ar), 7.16-7.05 (m, 4H, Ar), 6.45 (d, *J* = 3.2 Hz, 1H, Ar), 5.05-4.85 (m, 4H), 4.70 (m, 1H, CHCO), 2.75-2.70 (m, 2H, CH_2COOH); ^{13}C NMR (CD_3OD, 300 MHz) δ: 173, 169.2, 157, 153.7, 135, 130.3, 129.9, 125, 123.4, 121.5, 119.6, 119.48, 112, 111.8, 110.3, 110.1, 101.3, 74.8, 51, 48.9, 34.49; MS: m/z = 451.1 (M^+ + 1); HPLC purity: 97 % (area %).

(*S*)-3-(2-(5-Bromo-1*H*-indol-1-yl)acetamido)-5-(2,6-difluorophenoxy)-4-oxopentanoicacid (12b): Yield: (0.049 g, 42 %); m.p. 166-171 ºC; IR (KBr, ν_{max}, cm^{-1}): 3306, 2938, 1737, 1652, 1592, 1498, 1474, 1293; ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 8.75 (d, *J* = 8.2 Hz, 1H, NH), 7.72 (d, *J* = 0.5 Hz, 1H, Ar), 7.40-7.30 (m, 2H, Ar), 7.20-7.08 (m, 4H, Ar), 6.45-6.40 (d, *J* = 6.3 Hz, 1H, Ar), 5.05-4.80 (m, 4H), 4.70 (m, 1H, CHCO), 2.75-2.60 (m, 2H, CH_2COOH); ^{13}C NMR (CD_3OD, 300 MHz) δ: 173.3, 169, 157, 153.8, 135.29, 130.6, 129.9, 124.1, 123.47, 123.35, 122.8, 122.6, 112.5, 112, 111.89, 11.79, 110.6, 101.37, 74.9, 51.4, 48.9, 34.47; MS: m/z = 497.0 (M^+ + 2); HPLC purity: 94 % (area %).

(*S*)-5-(2,6-Difluorophenoxy)-3-(2-(5-fluoro-1*H*-indol-1-yl)acetamido)-4-oxopentanoicacid (12c): Yield: (0.064 g, 60 %); m.p. 174-177 ºC; IR (KBr, ν_{max}, cm^{-1}): 3315, 2966, 1732, 1698, 1661, 1592, 1491, 1297; ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 13.50-12.50 (brs, 1H, COOH), 8.70 (d, *J* = 9.1 Hz, 1H, NH), 7.45 (d, *J* = 8.2 Hz, 1H, Ar), 7.38-7.28 (m, 2H, Ar), 7.15-7.05 (m, 3H, Ar), 6.90 (t, 1H, Ar), 6.42 (d, *J* = 6.1 Hz, 1H, Ar), 5.05-4.80 (m, 4H), 4.80-4.70 (m, 1H, CHCO), 2.75-2.60 (m, 2H, CH_2COOH); ^{13}C NMR (CD_3OD, 400 MHz) δ: 174.39, 170.8, 160.5, 158.2, 155.6, 134.59, 131.9, 131.6, 130.5, 124.9, 113.4, 113.15, 111.38 (m), 110.9, 106.4, 106.2, 103, 76.14, 51.9, 50.1, 35.4; MS: m/z = 435.1 (M^+ + 1); HPLC purity: 95 % (area %).

(*S*)-5-(2,6-Difluorophenoxy)-3-(2-(5-methoxy-1*H*-indol-1-yl) acetamido)-4-oxopentanoicacid (12d): Yield: (0.013 g, 9 %); m.p. 70-71 ºC; ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 8.70-8.60 (brs, 1H, NH), 7.35-7.20 (m, 2H, Ar), 7.15-7.10 (m, 3H, Ar), 7.05 (d, *J* = 3.2 Hz, 1H, Ar), 6.70 (dd, *J* = 12.1 Hz, *J* = 3.3 Hz, 1H, Ar), 6.35 (d, *J* = 3.3 Hz, 1H, Ar), 5.10-4.80 (m, 4H), 4.70 (m, 1H, CHCO), 3.75 (s, 3H, OCH_3), 2.75-2.65 (m, 2H, CH_2COOH); MS: m/z = 447.1 (M^+ + 1).

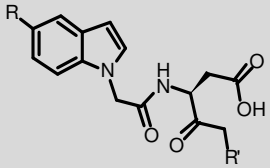
Caspase-3 inhibition assay: Caspase-3 fluorometric assay was used, which is based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC) by caspase-3, resulting in the release of the fluorescent 7-amido-4-methylcoumarin (AMC) moiety. Briefly, 5 µL of appropriate dilution of caspase-3 (caspase-3, human, recombinant, Sigma) were added to 5 µL of the tested inhibitors at various concentrations. The reaction was initiated by the addition of 90 µL of substrate to a final concentration of 10 µM in assay buffer (25 mM HEPES, 50 mM KCl, 0.1 % CHAPAS, 1 mM β-mercapto ethanol, pH 7.5 containing 2 mM DTT). Liberation of 7-amido-4-methyl coumarin was monitored continuously at room temperature (25 ºC) using a

victor V (Perkin Elmer, US) 96-well plate reader using an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Inhibitors stock solutions were prepared in DMSO and serial dilutions were made in DMSO. Controls were performed using enzyme alone, substrate alone, enzyme with DMSO and a positive control (IDN-6556). IC_{50} values were determined by non-linear regression analysis.

RESULTS AND DISCUSSION

The activity of the synthesized compounds to inhibit human recombinant caspase-3 was determined using a standard fluorometric assay[15-20] and the results are summarized in Table-1. The preliminary structure activity relationship of the synthesized compounds were carried out using IDN-6556 (IC_{50} = 0.06 μM) as reference standard. In order to understand the importance of the substituents in indole ring on the activity, we prepared compounds (**9a-d** and **12a-d**). Compound **9a**, with 2,3,5,6-tetrafluorophenoxy as warhead, had good activity with an IC_{50} value of 0.64 μM against caspase-3, which encouraged us to explore different substitution groups on indole ring for better active compounds. Compounds **9b-9c**, with halogens 5-Br and F in the indole ring were optimized and have shown similar activity as of **9a** indicating electron withdrawing nature of halogens, while **9d** was 2-fold less active than **9a**, indicating that electron donating group like 5-OCH_3 is least preferred with 2,3,5,6-tetrafluorophenoxy as warhead.

TABLE-1
CASPASE-3 ENZYME INHIBITION DATA[a]



Compound	R	R'	IC_{50} (μM)
9a	Cl	2,3,5,6-Tetrafluorophenoxy	0.64
9b	Br	2,3,5,6-Tetrafluorophenoxy	0.98
9c	F	2,3,5,6-Tetrafluorophenoxy	0.84
9d	OCH_3	2,3,5,6-Tetrafluorophenoxy	1.11
12a	Cl	2,6-Difluorophenoxy	> 20
12b	Br	2,6-Difluorophenoxy	1.20
12c	F	2,6-Difluorophenoxy	>20
12d	OCH_3	2,6-Difluorophenoxy	>20
IDN-6556*	–	–	0.06

*Standard reference. [a]Values are IC_{50} (μM) expressed as the mean of two replicate determinations.

We then explored the replacement of 2,3,5,6-tetrafluorophenoxymethyl ketone warhead by 2,6-difluorophenoxymethyl ketone with same substitution groups on indole ring to study preliminary structure activity relationship. Compound **12b** with 2,6-difluorophenoxymethyl ketone, have shown the activity with an IC_{50} value of 1.2 μM where as other compounds **12a**, **12c** and **12d** are less active with an IC_{50} value > 20 μM. Hence in terms of comparative studies with two warheads (2,3,5,6-tetrafluorophenoxy and 2,6-difluorophenoxy) the number of fluorine atoms present on the warhead greatly influenced the inhibition activity of caspase-3 with different indole derivatives explored.

Conclusion

A novel class of indole aspartyl ketones is synthesized and evaluated their activity as caspase-3 inhibitors. The preliminary SAR reveals that the activity of this series can be optimized by using 2,3,5,6-tetrafluorophenoxymethyl ketone as warhead and the limited diversity of the analogues discussed show that optimization of other substitution groups on indole ring will be interesting.

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