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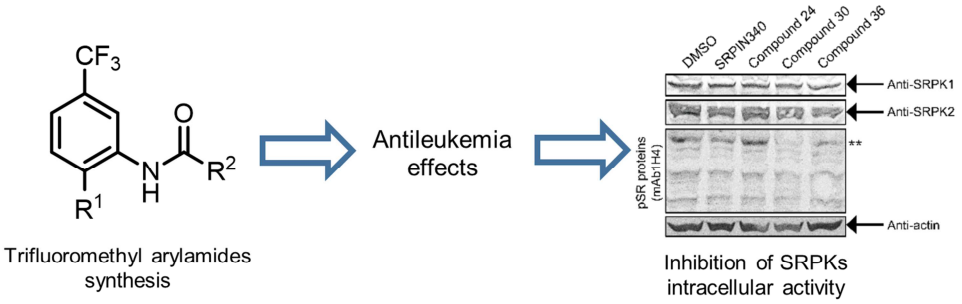

Trifluoromethyl arylamides synthesis

Inhibition of SRPKs intracellular activity

Trifluoromethyl arylamides with antileukemia effect and intracellular inhibitory activity over serine/arginine-rich protein kinases (SRPKs)

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Abstract

The serine/arginine-rich protein kinases (SRPKs) have frequently been found with altered activity in a number of cancers, suggesting they could serve as potential therapeutic targets in oncology. Here we describe the synthesis of a series of twenty-two trifluoromethyl arylamides based on the known SRPKs inhibitor *N*-(2-(piperidin-1-yl)-5-(trifluoromethyl)phenyl)isonicotinamide (SRPIN340) and the evaluation of their antileukemia effects. Some derivatives presented superior cytotoxic effects against myeloid and lymphoid leukemia cell lines compared to SRPIN340. In particular, compounds **24**, **30**, and **36** presented IC_{50} values ranging between 6.0 – 35.7 µM. In addition, these three compounds were able to trigger apoptosis and autophagy, and to exhibit synergistic effects with the chemotherapeutic agent vincristine. Furthermore, compound **30** was more efficient than SRPIN340 in impairing the intracellular phosphorylation status of SR proteins as well as the expression of MAP2K1, MAP2K2, VEGF, and RON oncogenic isoforms. Therefore, novel compounds with increased intracellular effects against SRPK activity were obtained, contributing to medicinal chemistry efforts towards the development of new anticancer agents.

Keywords

Trifluoromethyl arylamides, SRPK, SRPIN340, serine/arginine-rich protein kinase, leukemia, pre-mRNA splicing.

1. Introduction

Serine/arginine-rich protein kinases (SRPKs) are serine-threonine kinases related to the phospho-regulation of serine-arginine proteins (SR proteins), a protein family involved in pre-mRNA splicing control [1, 2]. Overexpression of the SRPK1 and SRPK2 family members has been related to tumorigenesis and to poor patient prognosis of many human cancers including leukemia [3, 4], colon [5, 6], pancreatic [6, 7], melanoma [8], breast [6, 9], prostate [10], and glioma [11]. In the intracellular context of cancerous cells, dysregulated SRPKs activity promotes cell proliferation and apoptosis escape [3, 12], suggesting that they are potential targets for the development of new anticancer agents [13, 14].

SRPKs have also been associated with the infection mechanisms of multiple viruses, including HIV, hepatitis, dengue, and Epstein-Barr virus [15-17]. Screening for SRPKs inhibitors with antiviral activity, Hagiwara and colleagues identified the isonicotinamide compound *N*-(2-(piperidin-1-yl)-5-(trifluoromethyl)phenyl) isonicotinamide (also called SRPIN340) (**Fig. 1**), which is able to selectively inhibit SRPK1 and SRPK2 [16].

Since the identification of SRPIN340, different studies have been conducted to evaluate its pharmacological potential in different *in vitro* and *in vivo* disease models, including viral infection [16, 18, 19], angiogenesis [20, 21], and cancer [8]. Within this context, in our previous studies we evaluated the cytotoxic potential of SRPIN340 in a panel of leukemia cells with high expression levels of SRPK1 and SRPK2. This compound was able to reduce cell viability, decrease hyperphosphorylation of SR family members (SRSF2, SRSF4, SRSF5 and SRSF6), and to regulate the expression of genes involved in cell proliferation and survival (MAP2K1, MAP2K2, VEGF and FAS) [4]. Recently, other SRPK inhibitors have also been described. Similar to SRPIN340, they displayed important biological effects (**Fig. 1**) [22, 23].

Even though these reports have indicated promising results for SRPK pharmacological inhibition in pre-clinical *in vitro* and *in vivo* assays, the search for novel compounds with increased biological efficiency is of potential interest [8]. Here we describe the design and synthesis of a series of twenty-two trifluoromethyl arylamides and the assessment of their potential antileukemia effects.

2. Results and discussion

2.1. Synthesis

Trifluoromethyl arylamide SRPIN340, as well as, compounds **15**-**36** were prepared in three steps. First, commercially available 1-fluoro-2-nitro-4-(trifluoromethyl)benzene (**1**) was treated with amines to obtain derivatives **2**-**7** with yields ranging 81% – 98% (**Scheme 1**).

After that, compounds **2** - **7** were submitted to reduction reactions with $SnCl_2$/HCl producing derivatives **8** - **13** (**Scheme 2**).

Finally, nucleophilic acyl substitution reactions (**Scheme 3, Table 1**), involving amines **8** - **13** and aromatic acyl chlorides, produced SRPIN340 (75% yield) and twenty-two other trifluoromethyl arylamides, compounds named **15 – 36** (30% – 91% yield). All synthesized compounds were fully characterized by infrared (IR) and nuclear magnetic resonance (NMR, ^{1}H and ^{13}C) spectroscopy techniques, as well as, by high resolution mass spectrometry (*vide infra*).

The synthesis of the compounds **15 - 36** was planned so that the influence on the biological activity of different groups attached to position **1** (see **Scheme 3** and **Table 1** for numbering) could be assessed. Thus, amines containing alicyclic, aliphatic and aromatic portions were chosen for the preparation of the compounds. In addition, we also decided to vary the type of aromatic group attached to the carbonyl functionality so that the impact of these modifications on biological activity could also be evaluated. Accordingly, four types of aromatic acyl chlorides were used in the preparation of the compounds **15 - 36**. In order to compare the biological effects of

each derivative with SRPIN340, a well known SRPK inhibitor, the latter was also synthesized.

2.2 Effect of compounds on cell viability

The cytotoxic activity of the synthesized trifluoromethyl arylamides **15 - 36** and SRPIN340 was evaluated at different concentrations (0 – 200 µM) over HL60, Jurkat, and Nalm6 human leukemic cell lines and the half-maximal inhibitory concentration (IC_{50}) for each compound was determined. As shown in **Table 1**, among the twenty-two trifluoromethyl arylamides synthesized, ten of them were active against at least one of the leukemia cell lines (IC_{50} < 100 µM). The compounds **24**, **30**, and **36** were the most active ones (IC_{50} 14.2 – 35.7 µM, 8.5 – 17.8 µM, and 6.0 – 33.8 µM, respectively) and presented superior cytotoxicity in comparison to the SRPK inhibitor SRPIN340 (IC_{50} 38.3 - 75.4 µM). Although further structure-activity relationship studies should be performed, initial observations suggest that the presence of the aryl bromide group in novel compounds may be associated with their superior activity. These aryl halide groups (including groups with bromide or iodide) have been frequently found in the structures of kinase inhibitors, including the anticancer agents trametinib and vandetanib [24].

In order to evaluate if the most active compounds affect non-tumor cells, primary peripheral blood mononuclear cells (PBMC) were obtained and used in cytotoxic assays. As shown in **Fig. 2**, PBMC cells were less sensitive to the treatments than the evaluated leukemia lineages (**Table 1**). Although compound **24** slightly reduced the lymphocytes viability at the dosage investigated, overall these compounds seem to be selective to leukemic cells.

2.3 Combinatorial effect with Vincristine

We further investigated potential interactions of compounds **24**, **30**, and **36** with vincristine, a component of many multi-drug pediatric and adult cancer chemotherapy, including leukemia [25]. For this purpose, Nalm6 was incubated for 48 h with two

different doses, in isolation or in combination of compound **24** (8.9 and 17.9 µM), compound **30** (4.3 and 8.5 µM), and compound **36** (1.5 and 3.0 µM) with vincristine (0.5 and 1.0 nM). These doses correspond to 25% and 50% of the IC_{50} value previously obtained for each compound (**Table 1**). After treatments, cell viability was measured and the combination index (CI) for each drug combination was calculated using the Chou-Talalay method [26]. According to this method, CI values significantly lower than 1.0 (CI < 1.0) indicate synergistic effect whereas values close to 1.0 indicate additive effect. Synergistic effects were observed for combinations containing lower concentrations of the compounds **24**, **30**, and **36** (i.e., 25% of the IC_{50}) as the calculated CI values were 0.57, 0.45, and 0.56, respectively (**Fig. 3**). Moreover, combinations performed in concentrations corresponding to 50% of the IC_{50} indicated synergism for compound **30** (CI = 0.78) but additive effect for compounds **24** and **36** (CI = 1.02 and CI = 1.05, respectively). Despite this apparent incongruence, this has been previously reported and seems to be related to the saturation of drug-target complexes at higher concentrations or due to some interactions between compounds [27], which is still unknown for our system. In addition, it is noteworthy that vincristine acts on a nanomolar scale while compounds **24**, **30**, and **36** act on a micromolar scale, resulting in dose-response curves with different maximum effects. Then, this can change the synergy to additive effect when drug concentrations are increased [28]. Nevertheless, the data obtained indicates that pharmaceutical formulations containing these compounds maybe approached to increase the potency of chemotherapeutic agents, mainly at lower dosages, which is the overall goal of such a strategy.

2.4 Effect of compounds on cell death and proliferation

Once compounds **24**, **30**, and **36** were selected as the most active derivatives, they were used in additional experiments in order to gain insights on how they might act in leukemic cells.

Annexin V/PI staining assays were performed to evaluate whether the treatments impact in Nalm6 apoptosis. After 12 or 24 h exposure, the three compounds significantly increased annexin-V positive cells in comparison to control (**Fig. 4A**). After 24 h of incubation, the percentage of cells in early events of apoptosis (annexin-V^+/PI^-) reached 11.9%, 14.6%, 24.7% when treated with compounds **24**, **30**, and **36,** respectively. Considering the percentage of total apoptotic cells (annexin-V^+/PI^- and annexin-V^+/PI^+), it was increased practically three times by treatment with compound **36** (**Fig. 4B**). Importantly, necrotic cells (annexin-V^-/PI^+), which is considered a toxic and degradative process of cell death [29], were barely noticed in these assays.

The effect of compounds on leukemic cells autophagy was also assessed by fluorescence microscopy. As shown in **Fig. 4C**, there was an increase in red fluorescence when Nalm6 cells were treated with 20 µM of the compounds during 24 h. These findings indicate the presence of autophagosomes and intracellular acidification in these cells, very similarly to the observed for cytarabine, a drug that acts on leukemic cells by triggering apoptosis and autophagy [30], which has been considered a complex cellular process that in some cases may increase cell death [31].

Finally, proliferation assays revealed that these three substances significantly impaired proliferation of HL60 and Nalm6 in a time-dependent manner (**Fig. 5**). After 96 h of incubation, compounds **24**, **30**, and **36** inhibited, respectively, 33%, 38%, and 48% of HL60 growth in comparison to control (**Fig. 5A**). Considering Nalm6, they inhibited cell growth in 37%, 66%, and 72%, respectively (**Fig. 5B**). Thus, these data suggest that pathways affecting cell proliferation are subjected to inhibition upon treatments. This should be the case of the SRPK2 related activity, as it has been described to promote leukemia cell proliferation in a previous study [3].

2.5. Effect on intracellular SRPKs activity

The effect of compounds in altering SRPKs intracellular activity was firstly evaluated by monitoring the expression pattern of transcripts already known to be

modulated by SRPKs [6, 21, 32]. With this approach, compound **30** was the most effective in impairing the expression of MAP2K1 and MAP2K2 as well as VEGF (**Fig. 6A**). Additionally, compounds **30** and **36** seemed to alter the splicing pattern of the apoptosis related gene RON. Interestingly, no clear changes in gene expression was observed in Nalm6 treated with SRPIN340, indicating the necessity of higher concentrations of this inhibitor at the experimental conditions used [4, 8]. No effects were observed in the expression pattern of the actin transcript, used here as endogenous loading control.

Intracellular activity of SRPKs was also monitored by checking the SR protein phosphorylation status through Western blotting assays. As shown in **Fig. 6B**, compound **30** was efficient in decreasing phospho-SR epitopes signals in Nalm6 lysates. Again, in the experimental condition used (treatments with 20 µM for 24 h), compound **30** was more efficient then the reference SRPK inhibitor SRPIN340. As controls, the expression of SRPK1, SRPK2 or actin proteins were checked but no difference was found during the treatments. These data suggest that we were able to obtain at least one compound with increased intracellular effect over SRPK activity, which the exact mechanism on SRPK inhibition *in vitro*, overall selectivity, membrane cell penetration, or *in vivo* effect in disease animal models deserve to be better elucidated in further studies.

3. Conclusions

A series of twenty-two trifluoromethyl arylamides were synthesized. Three compounds presented superior cytotoxicity against myelogenous and lymphoid leukemia cell lines as compared to the reference SRPK inhibitor SRPIN340. These three compounds impaired cell proliferation, presented synergistic effect in combination with the chemotherapeutic agent vincristine and were able to trigger apoptotic and autophagic cell death processes. Moreover, intracellular activity of SRPKs were affected by treatments with these compounds, mainly by compound **30**, which altered

MAP2K1, MAP2K2, VEGF, and RON gene expression as well as SR protein phosphorylation status. Therefore, these data collectively contribute to medicinal chemistry efforts towards the development of novel anticancer chemotherapeutic agents based on SRPK inhibition.

4. Experimental procedures

4.1 Synthetic procedures

4.1.1 Generalities

Analytical grade 1-fluoro-2-nitro-4-trifluoromethyl benzene, piperidine, morpholine, cyclohexylamine, diethylamine, 4-bromoaniline, pyrrolidine, isonicotinoyl chloride hydrochloride, nicotinoyl chloride hydrochloride, 2-chloropyridine-3-carboxylic acid and benzoyl chloride were purchased from Sigma Aldrich (St. Louis, MO, USA) and used without further purification. Anhydrous tin(II) chloride and triethylamine were purchased from Vetec (Rio de Janeiro, Brazil) and used as received. ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian Mercury 300 instrument at 300 MHz and 75 MHz, respectively, using $CDCl_3$ and CD_3OD as solvents. Infrared spectra were recorded on either a Varian 660-IR, equipped with GladiATR scanning from 4000 to 500 cm^{-1} or a Perkin Elmer Paragon 1000 FTIR spectrophotometer, using potassium bromide (1% v/v) disks, scanning from 600 to 4000 cm^{-1}. Melting points are uncorrected and were obtained with a MQAPF-301 melting point apparatus (Microquimica, Campinas, Brazil). Analytical thin layer chromatography was carried out on TLC plates covered with 60GF254 silica gel. Column chromatography was performed over silica gel (60–230 mesh). Solvents utilized as eluents were used without further purification.

*4.1.2 Synthesis of compounds **2** - **7***

*4.1.2.1 1-(2-nitro-4-(trifluoromethyl)phenyl)piperidine (**2**)*

A 100 mL round bottom flask initially placed in an ice bath was charged with 8.60 mL (88.2 mmol) of piperidine, 4.10 mL of dimethylformamide (DMF), and 4.20 mL (28.7

mmol) of 1-fluoro-2-nitro-4-trifluoromethyl benzene (**1**). The ice bath was removed and the resulting mixture was magnetically stirred at room temperature for 1.5 h. After this time, water was added and the resulting mixture was transferred to a separatory funnel. The aqueous phase was extracted with ethyl acetate (4 x 80 mL). The organic extracts were combined and the resulting organic layer was washed with brine, dried over sodium sulphate, filtered and concentrated under reduced pressure. The resulting solid was recrystallized with methanol. Compound **2** was obtained as an orange solid in 91% yield (7.15 g, 26.1 mmol).

TLC R_f = 0.40 (ethyl acetate - hexane 16:1 v/v). mp 50.1 - 50.7 ºC. IR (ATR, cm^{-1}) $\bar{\nu}_{max}$: 2938, 2867, 2827, 1621, 1560, 1528, 1493, 1449, 1386, 1323, 1297, 1260, 1233, 1211, 1149, 1115, 1080, 1064, 1021, 974, 929, 906, 882, 856, 832, 789, 760, 724, 678, 629, 528.^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.61-1.75 (m, 6H), 3.12 (t, 4H, J = 5.3 Hz), 7.14 (d, 1H, J = 8.7 Hz), 7.60 (dd, 1H, J = 8.7 Hz and J = 2.3 Hz), 8.03 (d, 1H,J = 2.3 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 24.0, 25.8, 52.3, 120.6, 120.9 (q, J_{C-F} = 34.1 Hz), 123.7 (q, J_{C-F} = 269.6 Hz), 124.6 (q, J_{C-F} = 4.0 Hz), 130.1 (q, J_{C-F} = 3.4 Hz), 139.8, 148.8. HRMS ($M+H^+$): Calculated for $C_{12}H_{14}F_3N_2O_2$, 275.1007; found: 275.0926.

Nitro compounds **3** - **7** (**Scheme 1**) were synthesized using a procedure similar to that described for the preparation of compound **2**. Description of experimental data that support the structures of compounds **3**-**7** is provided below.

4.1.2.2 N-cyclohexyl-2-nitro-4-(trifluoromethyl)aniline (**3**)

The compound was obtained as a yellow solid after recrystallization with methanol in 81% yield. TLC R_f = 0.10 (hexane). mp 79.3 - 80.2 ºC. IR (ATR, cm^{-1}) $\bar{\nu}_{max}$: 3365, 3114, 2931, 2861, 1634, 1572, 1529, 1436, 1411, 1324, 1260, 1244, 1227, 1187, 1152, 1112, 1063, 976, 912, 899, 831, 763, 694, 642 . ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.30-2.07 (m, 10H), 3.51-3.61 (m, 1H), 6.95 (d, 1H, J = 9.3 Hz), 7.56 (dd, 1H, J = 9.3 Hz and J = 2.1 Hz), 8.34 (d 1H, J = 6.3 Hz), 8.45 (d, 1H, J = 2.1 Hz).^{13}C NMR (75 MHz,

253 $CDCl_3$) δ: 24.6, 25.6, 32.7, 51.5, 115.0, 117.0 (q, J_{C-F}= 34.1 Hz), 123.9 (q, J_{C-F}= 269.0
254 Hz), 125.4 (q, J = 4.2 Hz), 132.1 (q, J = 3.0 Hz), 130.7, 146.2. HRMS ($M+H^+$):
255 Calculated for $C_{13}H_{15}F_3N_2O_2$, 289.1086; found: 289.0994.

256 *4.1.2.3 1-(2-nitro-4-(trifluoromethyl)phenyl)pyrrolidine* (**4**)

257 The compound was obtained as an orange solid in 97% after purification by silica
258 gel column chromatography eluted with hexane-ethyl acetate (5:1 v/v). TLC R_f = 0.45
259 (hexane-ethyl acetate 5:1 v/v). mp 52.3 - 53.8 ºC. IR (ATR, cm^{-1}) $\bar{\nu}_{max}$: 2975, 2871,
260 1622, 1554, 1504, 1428, 1388, 1322, 1268, 1150, 1103, 1074, 884, 808, 781, 719, 688,
261 634. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.98 - 2.03 (m, 4H), 3.23 - 3.27 (m, 4H), 6.95 (d, 1H,
262 J = 9.0 Hz), 7.53 (dd, 1H, J = 9.0 Hz and J = 2.4 Hz), 7.99 (brs, 1H). ^{13}C NMR (75 MHz,
263 $CDCl_3$) δ: 25.8, 50.8, 116.4, 117.1 (q, J_{C-F} = 34.2 Hz), 124.0 (q, J_{C-F} = 269.1 Hz), 124.7
264 (q, J_{C-F} = 4.0 Hz), 129.4 (q, J_{C-F} = 3.2 Hz), 135.8, 144.4. HRMS ($M+H^+$): Calculated for
265 $C_{11}H_{12}F_3N_2O_2$, 261.0851; found: 261.0770.

266 *4.1.2.4 N,N-diethyl-2-nitro-4-(trifluoromethyl)aniline* (**5**)

267 The compound was obtained as an orange oil in 98% yield after purification by
268 silica gel column chromatography eluted with hexane-ethyl acetate (8:1 v/v). TLC R_f =
269 0.55 (hexane - ethyl acetate 8:1 v/v). IR (ATR, cm^{-1}) $\bar{\nu}_{max}$: 2979, 2939, 2877, 1621,
270 1531, 1322, 1258, 1114, 1083, 903, 877, 816, 784, 717, 669, 601. ^{1}H NMR (300 MHz,
271 $CDCl_3$) δ: 1.16 (t, 6H, J = 7.1 Hz), 3.26 (q, 4H, J = 7.1 Hz), 7.14 (d, 1H, J = 9.0 Hz),
272 7.58 (dd, 1H, J = 9.0 Hz and J = 2.3 Hz), 7.96 (brs, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ:
273 12.6, 46.1, 119.9 (q, J_{C-F} = 34.0 Hz),120.6, 123.8 (q, J_{C-F} = 269.3 Hz), 124.5 (q, J_{C-F} =
274 4.0 Hz), 129.4 (q, J_{C-F} = 3.3 Hz), 139.8, 146.6. HRMS ($M+H^+$): Calculated for
275 $C_{11}H_{14}F_3N_2O_2$, 263.1007; found: 263.0944.

276 *4.1.2.5 4-(2-nitro-4-(trifluoromethyl)phenyl)morpholine* (**6**)

The compound was obtained as an orange oil in 97% yield after purification by silica gel column chromatography eluted with hexane-ethyl acetate (3:1 v/v). TLC R_f = 0.27 (hexane-ethyl acetate 3:1 v/v). IR (ATR, cm^{-1}) $\bar{\nu}_{max}$: 2967, 2858, 1713, 1622, 1532, 1322, 1275, 1252, 1235, 1168, 1110, 1083, 1044, 938, 884, 824, 789, 720, 678, 640, 526. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 3.13 (t, 4H, J = 4.7 Hz), 3.84 (t, 4H, J = 4.7 Hz), 7.16 (d, 1H, J = 8.7 Hz), 7.68 (dd, 1H, J = 8.7 Hz and J = 2.3 Hz), 8.05 (brs, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 51.4, 66.6, 120.6, 123.4 (q, J_{C-F} = 269.9 Hz), 122.9 (q, J_{C-F} = 34.1 Hz), 124.4 (q, J_{C-F} = 3.9 Hz), 130.5 (q, J_{C-F} = 3.3 Hz), 141.0, 148.1. HRMS ($M+H^+$): Calculated for $C_{11}H_{12}F_3N_2O_3$, 277.0800; found: 277.0727.

4.1.2.6 N-(4-bromophenyl)-2-nitro-4-(trifluoromethyl)aniline (**7**)

The compound was obtained as an orange solid in 93% yield after purification by silica gel column chromatography eluted with hexane - ethyl acetate (5:1 v/v). TLC R_f = 0.78 (hexane - ethylacetate 5:1 v/v). mp 89.5 - 89.9 ºC. IR (ATR, cm^{-1}) $\bar{\nu}_{max}$: 3347, 3102, 1636, 1571, 1528, 1486, 1430, 1319, 1250, 1147, 1070, 1011, 909, 841, 805, 693, 632. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.15-7.26 (m, 3H), 7.54-7.60 (m, 3H), 8.50 (brs, 1H), 9,63 (brs,1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 116.8, 120.1 (q, J_{C-F} = 34.1 Hz), 120.2, 123.5 (q, J_{C-F} = 269.5 Hz), 125.0 (q, J_{C-F} = 4.2 Hz), 126.8, 132.1 (q, J_{C-F} = 3.2 Hz), 132.4, 133.3, 136.8, 144.9. HRMS ($M+H^+$): Calculated for $C_{13}H_8BrF_3N_2O_2$, 359.9721; found: 359.9648.

4.1.3 *Synthesis of compounds **8** - **13***

4.1.3.1 2-(piperidin-1-yl)-5-(trifluoromethyl)aniline (**8**)

A 50 mL round bottom flask initially placed in an ice bath was charged with 10.8 mL (129.6 mmol) of concentrated hydrocloric acid, 6.71 g (35.4 mmol) of tin(II) chloride, 20.0 mL of methanol, and 1.50 g (5.47 mmol) of 1-(2-nitro-4-(trifluoromethyl)phenyl)piperidine (**2**). The ice bath was removed and the resulting mixture was continuously stirred at room temperature for 42 h. After this time, sodium

hydroxide solution was added to the mixture until pH was approximately equal to 10. Then, the mixture was transferred to a separatory funnel and extracted with ethyl acetate (4 x 80.0 mL). The organic extracts were combined and the resulting mixture was washed with brine, dried under sodium sulphate, filtered and concentrated under reduced pressure. The residue was purified by silica gel column chromatography eluted with hexane-ethyl acetate (11:1 v/v). The compound **8** was obtained as a white solid in 78% yield (1.34 g, 5.49 mmol).

TLC R_f = 0.48 (hexane-ethyl acetate 11:1 v/v). mp 50.0 - 50.5 ºC. IR (ATR, cm^{-1}) $\bar{\nu}_{max}$: 3452, 3355, 2950, 2865, 2805, 1611, 1589, 1512, 1469, 1439, 1379, 1328, 1288, 1256, 1227, 1205, 1160, 1104, 1064, 936, 892, 860, 810, 745, 722, 663, 643. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.60-1.75 (m, 6H), 2.88 (brs, 4H), 4.11 (brs, 2H, $N\underline{H}_2$), 6.93-7.03 (m, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 24.4, 26.8, 52.4, 111.5 (q, J_{C-F} = 3.5 Hz), 115.5 (q, J_{C-F} = 4.1 Hz), 119.7, 124.7 (q, J_{C-F} = 270.0 Hz), 126.1 (q, J_{C-F} = 31.9 Hz), 141.7, 143.4. HRMS ($M+H^+$): Calculated for $C_{12}H_{16}F_3N_2$, 245.1266; found: 245.1182.

The anilines **9** - **13** (**Scheme 2**) were synthesized from compounds **3**-**7** using a similar procedure to that described for the preparation of **8**. Description of experimental data that support the structures of compounds **9** - **13** is provided below.

4.1.3.2 N-cyclohexyl-4-(trifluoromethyl)benzene-1,2-diamine (**9**)

The compound was obtained as a white solid in 56% yield after purification by silica gel column chromatography eluted with hexane - ethyl acetate (14:1 v/v). TLC R_f = 0.25 (hexane-ethyl acetate 14:1 v/v). mp 71.6 - 72.0 ºC. IR (ATR, cm^{-1}) $\bar{\nu}_{max}$: 3421, 3350, 2928, 2855, 1625, 1601, 1528, 1470, 1440, 1362, 1324, 1300, 1240, 1217, 1146, 1107, 1084, 1055, 913, 885, 863, 808, 737, 668, 635. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.17 - 2.15 (m, 10H), 3.25 - 3.37 (m, 4H), 6.65 (d, 1H, J = 8.4 Hz), 6.93 (d, 1H, J = 1.8 Hz), 7.08 (dd, 1H, J = 8.4 Hz and J = 1.8 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 25.1, 26.0, 33.4, 51.8, 110.9, 113.9 (q, J_{C-F}= 3.7 Hz), 118.6 (q, J_{C-F}= 4.1 Hz), 119.3 (q, J_{C-F}= 32.1), 125.2

(q, J_{C-F}= 268.8 Hz), 133.2, 139.9. HRMS (M+H$^+$): Calculated for $C_{13}H_{18}F_3N_2$, 259.1422; found: 259.1341.

4.1.3.3 2-(pyrrolidin-1-yl)-5-(trifluoromethyl)aniline (**10**)

The compound was obtained as a red oil in 77% yield after purification by column chromatography eluted with hexane - ethyl acetate (3:1 v/v). TLC R_f = 0.68 (hexane - ethyl acetate 3:1 v/v). IR (ATR, cm^{-1}) $\bar{\nu}_{max}$: 3440, 3355, 2969, 2877, 2823, 1711, 1618, 1516, 1439, 1328, 1244, 1148, 1105, 954, 903, 866, 808, 661. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.91 - 1.97(m, 4H), 3.09-3.13 (m, 4H), 3.92 (brs, 2H), 6.92 - 6.98 (m, 3H).^{13}C NMR (75 MHz, $CDCl_3$) δ: 24.3, 50.6, 112.1 (q, J_{C-F} = 3.8 Hz), 115.8 (q, J_{C-F} = 4.1 Hz), 117,8, 124.8 (q, J_{C-F} = 269.6 Hz), 124.8 (q, J_{C-F} = 31.8 Hz, C-5), 140.8. HRMS (M+H$^+$): Calculated for $C_{11}H_{14}F_3N_2$, 231.1109; found: 231.1026.

4.1.3.4 N,N-diethyl-4-(trifluoromethyl)benzene-1,2-diamine (**11**)

The compound was obtained as a yellow oil in 67% yield after purification by column chromatography eluted with hexane - ethyl acetate (12:1 v/v). TLC R_f = 0.22 (hexane -ethyl acetate 30:1 v/v). IR (ATR, cm^{-1}) $\bar{\nu}_{max}$: 3469, 3362, 2973, 2933, 2870, 2826, 1615, 1593, 1514, 1441, 1384, 1335, 1294, 1260, 1232, 1163, 1120, 928, 867, 817, 745, 666. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 0.99 (t, 6H, J = 7.1 Hz), 2.99 (q, 4H, J = 7.1 Hz), 4.19 (brs, 2H), 6.94-6.97 (m, 2H), 7.05 (d, 1H, J = 8.7 Hz).^{13}C NMR (75 MHz, $CDCl_3$) δ: 12.3, 46.6, 111.3 (q, J_{C-F} = 3.7 Hz), 114.6 (q, J_{C-F} = 3.9 Hz), 122.5, 124.4 (q, J_{C-F} = 270.0 Hz), 126.3 (q, J_{C-F} = 31.8 Hz), 139.9, 143.7. HRMS (M+H$^+$): Calculated for: $C_{11}H_{16}F_3N_2$, 233.1266; found: 233.1211.

4.1.3.5 2-*morpholino-5-(trifluoromethyl)aniline* (**12**)

Compound was obtained as a white solid in 94% yield without any further purification. TLC R_f = 0.48 (hexane - ethylacetate 3:1 v/v). mp 130.6 - 131.1 ºC. IR (ATR, cm^{-1}) $\bar{\nu}_{max}$: 3430, 3338, 2827, 2823, 1620, 1515, 1448, 1331, 1256, 1217, 1153, 1099, 938, 897, 860, 818, 651. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 2.95 (t, 4H, J = 4.7 Hz), 3.87 (t, 4H, J = 4.7 Hz), 6.96 (brs, 1H), 6.96-7.05 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 51.2, 67.6, 111.9 (q, J_{C-F} = 3.7 Hz), 115.7 (q, J_{C-F} = 4.0 Hz), 119.7, 124.6 (q, J_{C-F} = 270.0 Hz), 126.9 (q, J_{C-F} = 32.0 Hz), 141.7. HRMS ($M+H^+$): Calculated for $C_{11}H_{14}F_3N_2O$, 247.1058; found: 247.0956.

4.1.3.6 N-(4-bromophenyl)-4-(trifluoromethyl)benzene-1,2-diamine (**13**)

Compound was obtained as a white solid in 79% yield after purification by silica gel column chromatography eluted with hexane-ethyl acetate (5:1 v/v). TLC R_f = 0.25 (hexane-ethyl acetate 5:1 v/v). mp 123.5-123.8 ºC. IR (ATR, cm^{-1}) $\bar{\nu}_{max}$: 3469, 3384, 1591, 1518, 1485, 1436, 1385, 1334, 1249, 1154, 1106, 928, 868, 820. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 3.73 (brs, 2H), 5.39 (brs, 1H), 6.72 (d, 2H, J = 9.0 Hz), 6.97-7.91 (m, 2H), 7.16 (d, 1H, J = 8.1 Hz), 7.34 (d, 2H, J = 9.0 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 113.0, 113.6 (q, J_{C-F} = 3.7 Hz), 117.0 (q, J_{C-F} = 3.9 Hz), 118.6, 121.9 (C-6), 124.4 (q, J_{C-F} = 270.3 Hz), 126.7 (q, J_{C-F} = 32.3 Hz), 132.5, 132.6, 139.5, 142.7.HRMS ($M+H^+$): Calculated for $C_{13}H_{11}BrF_3N_2$, 331.0058; found: 330.9987.

*4.1.4 Synthesis of SRPIN340 and compounds **15** - **36***

4.1.4.1 N-(2-(piperidin-1-yl)-5-(trifluoromethyl)phenyl)isonicotinamide (*SRPIN340*)

A 25 mL round bottom flask initially placed in an ice bath was charged with 0.629 g (3.389 mmol) of isonicotinoyl chloride hydrochloride, 0.800 mL of triethylamine, 8.00 mL of dichoromethane and 0.400 (1.64 mmol) of 2-(piperidin-1-yl)-5-(trifluoromethyl) aniline (**8**). The ice-bath was removed and the mixture was magnetically stirred at room temperature for 3 h. Then, 10.0 mL of distilled water was added, and the mixture was transferred to a separatory funnel. The aqueous layer was extracted with ethyl acetate

(4 x 30.0 mL). The organic extracts were combined and the resulting organic layer was washed with brine, dried over sodium sulphate, filtered, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography eluted with hexane-ethyl acetate (3:1 v/v). The solid was further recrystallized with acetone. The compound SRPIN340 was obtained as a white solid in 75% yield (430 mg, 1.23 mmol).

TLC R_f = 0.13 (hexane - ethyl acetate 3:1 v/v). mp 95.6 - 96.7 ºC. IR (ATR, cm^{-1}) $\bar{\nu}_{max}$: 3347, 2945, 2917, 2811, 1679, 1611, 1587, 1556, 1527, 1455, 1434, 1380, 1334, 1308, 1239, 1165, 1107, 1093, 1061, 1022, 915, 895, 878, 839, 826, 751, 728, 681, 662, 644. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.65 - 1.81 (m, 6H), 2.86 (t, 4H, J = 5.1 Hz), 7.28 (d, 1H, J = 8.4 Hz), 7.37 (dd, 1H, J = 8.4 Hz and J = 1.8 Hz), 7.76 (dd, 2H, J = 4.5 Hz and J = 1.5 Hz), 8.83 - 8.85 (m, 3H), 9.55 (s, 1H, NH). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 24.0, 27.1, 53.8, 116.6, 120.8, 121.1, 121.6 (q, J_{C-F} = 3.7 Hz), 124.2 (q, J_{C-F} = 270.5 Hz), 127.5 (q, J_{C-F} = 32.3 Hz), 133.4, 141.8, 145.9, 151.1, 163.0. HRMS ($M+H^+$): Calculated for $C_{18}H_{19}F_3N_3O$, 350.1480; found: 350.1420.

The trifluoromethyl amides **15** - **36** (**Scheme 3**) were prepared by using a similar methodology to that described for the synthesis of SRPIN340. Description of experimental data that support the structures of compounds **15 - 36** is provided below.

4.1.4.2 N-(2-(cyclohexylamino)-5-(trifluoromethyl)phenyl)isonicotinamide (**15**)

The compound was obtained as a white solid in 82% yield after recrystallization with ethyl acetate. TLC R_f = 0.33 (hexane - ethyl acetate 1:1 v/v). mp 159.9 -160.2 ºC. IR (ATR, cm^{-1}) $\bar{\nu}_{max}$: 3262, 2931, 2851, 1657, 1617, 1543, 1510, 1485, 1441, 1324, 1205, 1254, 1238, 1147, 1133, 1103, 1069, 998, 931, 880, 841, 806, 754, 709, 687, 637. ^{1}H NMR (300 MHz, CD_3OD) δ: 1.10 – 2.20 (m, 10H), 3.30 (quint, 1H, J = 1.8 Hz), 3.32 - 3.43 (m, 1H), 6.87 (d, 1H, J = 8.7 Hz), 7.40 (dd, 1H, J = 8.7 Hz and J = 1.7 Hz), 7.47-7.46 (m, 1H), 7.93 (dd, 2H, J = 4.7 Hz and J = 1.8 Hz), 8.73 (dd, 2H, J = 4.7 Hz

and J = 1.8 Hz).^{13}C NMR (75 MHz, CD_3OD) δ: 24.9, 25.7, 32.6, 51.3, 111.2, 116.9 (q, J_{C-F}= 32.7 Hz), 121.6, 122.1, 124.6 (q, J = 3.8 Hz), 125.1 (q, J_{C-F} = 3.9 Hz), 125.1 (q, J = 267.7 Hz), 142.4, 145.8, 149.7, 165.9. HRMS ($M+H^+$): Calculated for $C_{19}H_{21}F_3N_3O$, 364.1637; found: 364.1556.

4.1.4.3 N-(2-(pyrrolidin-1-yl)-5-(trifluoromethyl)phenyl)isonicotinamide (**16**)

The compound was obtained as a white solid in 70% yield after recrystallization with acetone. TLC R_f = 0.24 (hexane - ethyl acetate 1:1 v/v). mp 110.0 - 110.6 ºC. IR (ATR, cm^{-1}) $\bar{\nu}_{max}$: 3242, 2976, 2872, 1654, 1613, 1538, 1512, 1489, 1436, 1409, 1370, 1327, 1291, 1152, 1093, 929, 901, 849, 816, 755, 656. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.94 - 1.98 (m, 4H), 3.13 - 3.17 (m, 4H), 7.10 (d, 1H, J = 8.7 Hz), 7.35 (dd, 1H, J = 8.7 Hz and J = 1.8 Hz), 7.71 - 7.73 (m, 2H), 8.31 (brs, 1H), 8.77 (brs, 2H), 8.97 (brs, 1H).^{13}C NMR (75 MHz,$CDCl_3$) δ: 25.0, 51.9, 118.4, 121.0, 121.1, 123.0 (q. J_{C-F} = 3.6 Hz), 124.4 (q, J_{C-F} = 269.9 Hz), 124.3 (q, J_{C-F} = 32.6 Hz), 129.3, 141.7, 145.1, 150.9, 163.5. HRMS ($M+H^+$): Calculated for $C_{17}H_{17}F_3N_3O$, 336.1324; found: 336.1282.

4.1.4.4 N-(2-(diethylamino)-5-(trifluoromethyl)phenyl)isonicotinamide (**17**)

The compound was obtained as a white solid in 85% yield after purification by silica gel column chromatography eluted with hexane-ethyl acetate (2:1 v/v). TLC R_f = 0.60 (hexane - ethyl acetate 1:1 v/v). mp 73.8 - 74.3 ºC. IR (ATR, cm^{-1}) $\bar{\nu}_{max}$: 3326, 2976, 2925, 2856, 1680, 1588, 1530, 1439, 1333, 1241, 1164, 1094, 1060, 922, 895, 826, 746, 676, 562. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 0.98 (t, 6H, J = 7.2 Hz), 3.02 (q, 4H, J = 7.2 Hz), 7.31 - 7.40 (m, 2H), 7.72 (dd, 2H, J = 4.5 Hz and J = 1.5 Hz), 8.82 - 8.89 (m, 3H), 9.92 (brs, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 13.0, 49.5, 116.3, 120.9, 121.7 (q, J_{C-F} = 3.7 Hz), 123.7, 124.1 (q, J_{C-F} = 270.7 Hz), 128.3 (q, J_{C-F} = 32.8 Hz), 136.2, 141.9, 142.7, 151.0, 163.0. HRMS ($M+H^+$): Calculated for $C_{17}H_{19}F_3N_3O$, 338.1480; found: 338.1453.

4.1.4.5 N-(2-morpholino-5-(trifluoromethyl)phenyl)isonicotinamide (**18**)

The compound was obtained as a white solid in 78% yield after purification by silica gel column chromatography eluted with hexane-ethyl acetate (2:1 v/v). TLC R_f = 0.18 (hexane - ethyl acetate 2:1 v/v). mp 166.5 - 168.4 ºC. IR (ATR, cm^{-1}) $\bar{\nu}_{max}$: 3351, 2969, 2921, 2858, 1676, 1590, 1531, 1439, 1333, 1242, 1155, 1108, 918, 823, 750, 656. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 2.95 (t, 4H, J = 4.5 Hz), 3.91 (t, 4H, J = 4.5 Hz), 7.32 (d, 1H, J = 8.4 Hz), 7.41 (dd, 1H, J = 8.4 Hz and J = 2.1 Hz), 7.74 (dd, 2H, J = 4.5 Hz and J = 2.8 Hz), 8.85 - 8.86 (m, 3H), 9.48 (brs, 1H).^{13}C NMR (75 MHz, $CDCl_3$) δ: 52.6, 67.7, 117.0 (q, J_{C-F} = 3.9 Hz), 120.8, 121.3, 121.9 (q, J_{C-F} = 3.8 Hz), 124.0 (q, J_{C-F} = 270.6 Hz), 128.3 (q, J_{C-F} = 32.6 Hz), 133.5, 141.8, 144.2, 151.0, 162.9. HRMS ($M+H^+$): Calculated for $C_{17}H_{17}F_3N_3O_2$, 351.1273; found: 352.1218.

4.1.4.6 N-(2-(4-bromophenylamino)-5-(trifluoromethyl)phenyl)isonicotinamide (**19**)

The compound was obtained as a yellow solid in 81% yield after purification by silica gel column chromatography eluted with hexane-ethyl acetate (1:1 v/v). TLC R_f = 0.22 (hexane-ethylacetate 2:1 v/v). mp 203.5 - 203.9 ºC. IR (ATR, cm^{-1}) $\bar{\nu}_{max}$: 3386, 3243, 3081, 1675, 1589, 1510, 1469, 1324, 1249, 1163, 1101, 923, 885, 821, 804, 749. ^{1}H NMR (300 MHz, CD_3OD) δ: 7.06 (d, 2H, J = 8,7 Hz), 7.36-7.49 (m, 4H), 7.77-7.85 (m, 3H). 8.16 (brs, 1H), 8.75-8.77 (m, 2H), 10.14 (brs, 1H). ^{13}C NMR (75 MHz, CD_3OD) δ: 113.4, 117.5, 120.6 (q, J_{C-F} = 32.2 Hz), 121.8, 122.5, 124.5 (q, J_{C-F} = 3.8 Hz), 125.1 (q, J_{C-F} = 269.2 Hz), 125.3 (q, J_{C-F} = 3.8 Hz), 127.0, 132.6, 142.0, 142.3, 150.8, 165.2. HRMS ($M+H^+$): Calculated for $C_{19}H_{14}BrF_3N_3O$, 436.0272; found: 436.0202.

4.1.4.7 2-chloro-N-(2-(piperidin-1-yl)-5-(trifluoromethyl)phenyl)nicotinamide (**20**)

The compound was obtained as a white solid in 78% yield after purification by column chromatography eluted with hexane-ethyl acetate (3:1 v/v). mp 120.3 - 121.2 ºC. IR (ATR, cm^{-1}) $\bar{\nu}_{max}$: 3322, 2919, 2827, 1678, 1655, 1613, 1578, 1526, 1474, 1433, 1400, 1333, 1263, 1214, 1100, 915, 893, 858, 824, 754, 662, 642, 601 . ^{1}H NMR (300

MHz, $CDCl_3$) δ: 1.58 - 1.73 (m, 6H), 2.85 (t, 4H, *J* = 5.0 Hz), 7.30 (d, 1H, *J* = 8.4 Hz), 7.36 - 7.46 (m, 2H), 8.23 (dd, 1H, *J* = 8.4 Hz and *J* = 1.8 Hz), 8.54 (dd, 1H, *J* = 4.5 Hz and *J* = 1.8 Hz), 8.87 (s, 1H), 9.73 (brs, 1H).^{13}C NMR (75 MHz, $CDCl_3$) δ: 23.9, 26.6, 54.1, 116.8 (q, J_{C-F} = 3.7 Hz), 121.4, 121.7, 121.7 (q, J_{C-F} = 3.9 Hz), 123.2, 124.2 (q, J_{C-F} = 270.4 Hz), 127.6 (q, J_{C-F} = 32.1 Hz), 131.6, 133.8, 140.4, 146.1, 146.9, 151.6, 162.7. HRMS ($M+H^+$): Calculated for $C_{18}H_{18}ClF_3N_3O$, 384.1090; found: 384.1043.

4.1.4.8 2-chloro-N-(2-(pyrrolidin-1-yl)-5-(trifluoromethyl)phenyl)nicotinamide (**21**)

The compound was obtained as a white solid in 79% yield after purification by silica gel column chromatography eluted with hexane-ethyl acetate (2:1 v/v). TLC R_f = 0.52 (hexane - ethyl acetate 2:1 v/v). mp 147.5 - 148.7 ºC. IR (ATR, cm^{-1}) $\bar{\nu}_{max}$: 3232, 2968, 2882, 2818, 1659, 1615, 1581, 1535, 1508, 1405, 1368, 1331, 1275, 1151, 1095, 818, 802, 768, 708, 540. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.94 - 2.01 (m, 4H), 3.13 (t, 4H, *J* = 6.3 Hz), 7.20 (d, 1H, *J* = 8.7 Hz), 7.38 (dd, 1H, *J* = 8.7 Hz and *J* = 1.5 Hz), 7.44 (dd, 1H, *J* = 7.8 Hz and *J* = 4.7 Hz), 8.32 (dd, 1H, *J* = 7.8 Hz and *J* = 1.8 Hz), 8.79 (dd, 1H, *J* = 4.7 Hz and *J* = 1.8 Hz), 8.56 (d, *J* = 1.5 Hz, 1H), 9.32 (brs, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 24.8, 52.5, 119.3 (q, J_{C-F} = 3.8 Hz), 119.5, 122.6 (q, J_{C-F} = 3.7 Hz), 123.4, 125.3 (q, J_{C-F} = 32.5 Hz), 131.1, 131.3, 140.9, 144.7, 147.0 , 151.7, 162.6. The signal of the carbon of the CF_3 group presented low intensity and it was not noticed in the spectrum. HRMS ($M+H^+$): Calculated for $C_{17}H_{16}ClF_3N_3O$, 370.0934; found: 370.0851.

4.1.4.9 2-chloro-N-(2-(diethylamino)-5-(trifluoromethyl)phenyl)nicotinamide (**22**)

The compound was obtained as a yellow solid in 59% yield after purification by silica gel column chromatography eluted with hexane - ethyl acetate (3:1 v/v). TLC R_f = 0.75 hexane - ethyl acetate (1:1 v/v). mp 74.3 - 75.4 ºC. IR (ATR, cm^{-1}) $\bar{\nu}_{max}$: 3291, 2976, 2934, 2848, 1666, 1612, 1578, 1531, 1395, 1334, 1258, 1167, 1116, 1065, 926, 899, 829, 760, 696. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 0.96 (t, 6H, *J* = 7.1 Hz), 3.00 (q, 4H, *J* = 7.1 Hz), 7.31 (d, 1H, *J* = 8.4 Hz), 7.38 - 7.40 (m, 2H), 8.25 (dd, 1H, *J* = 7.7 Hz and *J*

= 2.0 Hz), 8.53 (dd, 1H, *J* = 4.7 Hz and *J* = 2.0 Hz), 8.92 (brs, 1H), 10.02 (brs, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 12.5, 49.1, 116.7 (brs), 121.3 (q, J_{C-F} = 3.8 Hz), 123.2, 123.9, 124.2 (q, J_{C-F} = 270.8 Hz), 131.5, 136.4, 140.5, 147.0, 151.6, 162.7. Signal for the carbon attached to CF_3 was of low intensity and it is not observed. The signal for the carbon attached to the chlorine as well as the signal for the aromatic carbon attached to the $-N(Et)_2$ presented the same chemical shift. HRMS ($M+H^+$): Calculated for $C_{17}H_{18}ClF_3N_3O$, 372.1090; found: 372.1016.

4.1.4.10 2-chloro-N-(2-morpholino-5-(trifluoromethyl)phenyl)nicotinamide (**23**)

The compound was obtained as a yellow solid in 91% yield after purification by column chromatography eluted with hexane-ethyl acetate (3:1 v/v). TLC R_f = 0.43 hexane - ethyl acetate (1:1 v/v). mp 131.2 - 133.2 ºC. IR (ATR, cm^{-1}) $\bar{\nu}_{max}$: 3258, 2924, 2890, 2844, 1665, 1616, 1581, 1539, 1489, 1440, 1400, 1329, 1268, 1108, 923, 895, 828, 807, 754, 648. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 2.94 (t, 4H, *J* = 4.5 Hz), 3.87 (t, 4H, *J* = 4.5 Hz), 7.36 (d, 1H, *J* = 8.4 Hz), 7.42-7.48 (m, 2H), 8.28 (dd, 1H, *J* = 7.8 Hz and *J* = 1.8 Hz), 8.55 (dd, 1H, *J* = 4.8 Hz and *J* = 1.8 Hz), 8.92 (brs, 1H), 9,82 (brs, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 52.9, 67.3, 117.3 (q, J_{C-F} = 3.8 Hz), 121.8, 123.4, 124.1 (q, J_{C-F} = 270.5 Hz), 128.5 (q, J_{C-F} = 32.6 Hz), 131.2, 134.1, 140.9, 144.3, 146.6, 151.8, 162.7. HRMS ($M+H^+$): Calculated for $C_{17}H_{16}ClF_3N_3O_2$, 386.0883; found: 386.0842.

4.1.4.11 N-(2-(4-bromophenylamino)-5-(trifluoromethyl)phenyl)-2-chloronicotinamide (**24**)

The compound was obtained as a yellow solid in 37% yield after recrystallization with acetone. TLC R_f = 0.58 (hexane - ethyl acetate 1:1 v/v). mp 175.0 - 176.0 ºC. IR (ATR, cm^{-1}) $\bar{\nu}_{max}$: 3404, 3217, 3048, 1644, 1592, 1529, 1489, 1401, 1334, 1098, 1073, 882, 808, 751. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 6,10 (brs, 1H), 6.81 (d, 2H, *J* = 8.7 Hz), 7.34 - 7.45 (m, 5H), 8.11 (s, 1H), 8.16 (dd, 1H, *J* = 7.8 Hz and *J* = 1.8 Hz), 8.49 (dd, 1H, *J* = 4.7 Hz and *J* = 2.0 Hz), 8.66 (brs, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 114.5, 120.0, 121.3, 121.7 (q, J_{C-F} = 3.8 Hz), 123.2, 124.2 (q, J_{C-F} = 3.8 Hz), 125.6 (q, J_{C-F} =

32.8 Hz), 129.0, 130.5, 132.7, 138.9, 140.4, 141.9, 147.1, 151.9, 163.6. HRMS ($M+H^+$): Calculated for $C_{19}H_{13}BrClF_3N_3O$, 469.9883; found: 469.9707.

4.1.4.12 N-(2-(piperidin-1-yl)-5-(trifluoromethyl)phenyl)nicotinamide (**25**)

The compound was obtained as a white solid in 65% yield after purification by silica gel column chromatography eluted with hexane-ethyl acetate (1:1 v/v). TLC R_f = 0.45 (hexane - ethyl acetate 1:1 v/v). mp 129.8 - 130.3 ºC. IR (ATR, cm^{-1}) $\bar{\nu}_{max}$: 3332, 2940, 2856, 2811, 1664, 1588, 1529, 1467, 1435, 1332, 1243, 1163, 1105, 1023, 893, 834, 729, 703, 645, 584. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.64 - 1.65 (m, 2H), 1.75 - 1.82 (m, 4H), 2.88 (t, 4H, J = 4.8 Hz), 7.28 (d, 1H, J = 8.4 Hz), 7.37 (dd, 1H, J = 8.4 Hz and J = 1.8 Hz), 7.50 (ddd, 1H, J = 7.8 Hz, J = 4.8 Hz and J = 0.8 Hz), 8.30 (dt, 1H, J = 7.8 Hz and J = 1.8 Hz), 8.80 (dd, 1H, J = 4.8 Hz and J = 1.8 Hz), 8.84 (brs, 1H), 9.15 (d, 1H, J = 1.8 Hz), 9.55 (brs, 1H).^{13}C NMR (75 MHz, $CDCl_3$) δ: 23.9, 27.0, 53.9, 116.7 (brs), 121.0, 121.4 (q, J_{C-F} = 3.8 Hz), 124.1, 124.2 (q, J_{C-F} = 270.6 Hz), 127.6 (q, J_{C-F} = 33.4 Hz), 130.6, 133.6, 135.6, 145.8, 147.7, 152.8, 163.2. HRMS ($M+H^+$): Calculated for $C_{18}H_{19}F_3N_3O$, 350.1480; found: 350.1396.

4.1.4.13 N-(2-(cyclohexylamino)-5-(trifluoromethyl)phenyl)nicotinamide (**26**)

The compound was obtained as a white solid in 53% yield after purification by silica gel column chromatography eluted with hexane-ethyl acetate (1:1 v/v). TLC R_f = 0.38 (hexane - ethylacetate 1:1 v/v). mp 137.0 - 138.4 ºC. IR (ATR, cm^{-1}) $\bar{\nu}_{max}$: 3434, 3234, 3046, 2930, 2852, 1643, 1615, 1591, 1532, 1456, 1331, 1105, 883, 813, 712, 636. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.12 - 2.03 (m, 10H), 3.28 - 3.29 (m, 1H), 4.19 (brs, 1H), 6.79 (d, 1H, J = 8.4 Hz), 7.36-7.46 (m, 2H), 7.52 (brs, 1H), 8.19 - 8.25 (m, 2H), 8.72 (d, 1H, J = 4.2 Hz), 9.08 (brs, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 24.7, 25.6, 32.9, 51.6, 112.5, 118.5 (q, J_{C-F}= 32.6 Hz), 124.4 (q, J = 269.1 Hz), 122.0, 123.7, 125.1,

129.6, 135.7, 144.6, 147.9, 152.6, 164.5. HRMS ($M+H^+$): Calculated for $C_{19}H_{21}F_3N_3O$, 364.1638; found: 364.1549.

4.1.4.14 N-(2-(pyrrolidin-1-yl)-5-(trifluoromethyl)phenyl)nicotinamide (**27**)

The compound was obtained as a white solid in 73% yield after recrystallization with ethyl acetate. TLC R_f = 0.33 (hexane - ethyl acetate 1:2 v/v). mp 127.8 - 128.5 ºC. IR (ATR, cm^{-1}) $\bar{\nu}_{max}$: 3289, 3056, 2972, 2870, 2842, 1644, 1615, 1592, 1530, 1372, 1332, 1266, 1249, 1152, 1109, 1082, 1024, 932, 897, 875, 827, 707, 656. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.96 - 2.01 (m, 4H), 3.20 (t, 4H, *J* = 6.2 Hz), 7.14 (d, 1H, *J* = 8.4 Hz), 7.35 - 7.38 (m, 1H), 7.48 (dd, 1H, *J* = 7.8 Hz and 4.8 Hz), 8.26 (dt,1H, *J* = 7.8 Hz and *J* = 1.8 Hz), 8.47 (brs, 1H), 8.79 (dd, 1H, *J* = 4.8 Hz and *J* = 1.8 Hz), 8.82 (brs, 1H), 9.10 (d,1H, *J* = 1.8 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 24.9, 52.2, 118.8, 119.6 (brs), 122.5 (q, J_{C-F} = 3.6 Hz), 124.4 (q, J_{C-F} = 270.2 Hz), 124.9 (q, J_{C-F} = 32.6 Hz), 124.1, 130.5, 130.6, 135.6, 144.8, 147.8, 152.9, 163.6. HRMS ($M+H^+$): Calculated for $C_{17}H_{17}F_3N_3O$, 336.1324; found: 336.1201.

4.1.4.15 N-(2-(diethylamino)-5-(trifluoromethyl)phenyl)nicotinamide (**28**)

The compound was obtained as a white solid in 87% yield after purification by silica gel column chromatography eluted with hexane - ethyl acetate (1:1 v/v). TLC R_f = 0.50 (hexane - ethyl acetate 1:1 v/v). mp 64.8 - 66.8 ºC. IR (ATR, cm^{-1}) $\bar{\nu}_{max}$: 3334, 2975, 2932, 2854, 1678, 1614, 1586, 1534, 1483, 1440, 1336, 1247, 1167, 1150, 1114, 1062, 1021, 923, 898, 828, 716, 567. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 0.99 (t, 6H, *J* = 7.2 Hz), 3.05 (q, 4H, *J* = 7.2 Hz), 7.32 (d, 1H, *J* = 8.1 Hz), 7.39 (dd, 1H, *J* = 8.1 Hz and *J* = 1.5 Hz), 7.49 (dd, 1H, *J* = 8.3 Hz and *J* = 4.7 Hz), 8.27 (dt, 1H, *J* = 8.3 Hz and *J* = 1.8 Hz), 8.80 (dd, 1H, *J* = 4.8 Hz and *J* = 1.8 Hz), 8.92 (d, 1H, *J* = 1.5 Hz), 9.12 (d, 1H, *J* =

1.8 Hz), 9.90 (brs, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 13.0, 49.5, 116.3 (q, J_{C-F} = 3.9 Hz), 121.0 (q, J_{C-F} = 3.9 Hz), 123.6, 124.1, 124.2 (q, J_{C-F} = 270.5 Hz), 128.3 (q, J_{C-F} = 32.3 Hz), 130.6, 135.6, 136.5, 142.6, 147.8, 152.9, 163.2. HRMS ($M+H^+$): Calculated for $C_{17}H_{19}F_3N_3O$, 338.1480; found: 338.1399.

4.1.4.16 N-(2-morpholino-5-(trifluoromethyl)phenyl)nicotinamide (**29**)

The compound was obtained as a white solid in 74% yield after purification by silica gel column chromatography eluted with hexane - ethyl acetate (1:1 v/v). TLC R_f = 0.18 (hexane - ethyl acetate 1:1 v/v). 157.5 - 159.0 mp ºC. IR (ATR, cm^{-1}) $\bar{\nu}_{max}$: 3344, 2970, 2846, 1674, 1588, 1534, 1469, 1441, 1339, 1247, 1198, 1156, 1114, 1022, 936, 918, 897, 880, 833, 734, 707, 661. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 2.95 (t, 4H, J = 4.5 Hz), 3.91 (t, 4H, J = 4.5 Hz), 7.33 (d, 1H, J = 8.1 Hz), 7.43 (dd, 1H, 8.4 Hz and J = 2.1 Hz), 7.51 (dd, 1H, J = 7.8 Hz and J = 4.8 Hz), 8.28 (td, 1H, J = 7.8 Hz and 1.8 Hz), 8.81 - 8.88 (m, 2H), 9,13 (brs, 1H), 9.45 (brs, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 52.6, 67.7, 117.0 (q, J_{C-F} = 3.9 Hz), 121.3, 121.6 (q, J_{C-F} = 3.7 Hz), 124.1 (q, J_{C-F} = 270.3 Hz), 124.2, 128.4 (q, J_{C-F} = 32.5 Hz), 130.4, 133.7, 135.5, 144.1, 147.6, 153.1, 163,1. HRMS ($M+H^+$): Calculated for $C_{17}H_{17}F_3N_3O_2$ 352.1273; found: 352.1201.

4.1.4.17 N-(2-(4-bromophenylamino)-5-(trifluoromethyl)phenyl)nicotinamide (**30**)

The compound was obtained as a white solid in 30% yield after recrystallization with acetone. TLC R_f = 0.38 (hexane - ethyl acetate 1:1 v/v). mp 166.7 - 167.2 ºC. IR (ATR, cm^{-1}) $\bar{\nu}_{max}$: 3314, 3188, 3068, 1663, 1621, 1592, 1514, 1440, 1337, 1250, 1162, 1114, 1074, 1025, 1008, 887, 808, 709. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 6.34 (brs, 1H), 6.82 (d, 2H, J = 8.7 Hz), 7.33 - 7.46 (m, 5H), 8.08 – 8.12 (m, 2H), 8.72 (dd, 1H, J = 4.8 Hz and J = 1.5 Hz), 8.96 (d, 1H, J = 1.5 Hz), 8.53 (brs, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 114.4, 120.0, 121.3, 121.6 (q, J_{C-F} = 3.6 Hz), 123.9 (q, J_{C-F} = 3.7 Hz), 124.1, 124.1 (q, J_{C-F} = 270.0 Hz), 125.5 (q, J_{C-F} = 33.4 Hz), 129.2, 129.7, 132.7, 135.8, 138.9, 141.9,

148.0, 152.9, 164.5. HRMS ($M+H^+$): Calculated for $C_{19}H_{14}BrF_3N_3O$, 436.0272; found: 436.0200.

4.1.4.18 N-(2-(piperidin-1-yl)-5-(trifluoromethyl)phenyl)benzamide (**31**)

The compound was obtained as a white solid in 88% yield after purification by silica gel column chromatography eluted with hexane-ethyl acetate (5:1 v/v). TLC R_f = 0.55 (hexane - ethyl acetate 5:1 v/v). mp 116.0 - 117.0 ºC. IR (ATR, cm^{-1}) $\bar{\nu}_{max}$: 3338, 2932, 2846, 1675, 1588, 1530, 1472, 1439, 1378, 1337, 1272, 1240, 1162, 1116, 1026, 932, 913, 902, 877, 828, 796, 697, 648. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.64 - 1.66 (m, 2H), 1.74 - 1.82 (m, 4H), 2.88 (t, 4H, J = 5.1 Hz), 7.26 (d, 1H, J = 8.1 Hz), 7.35 (d, 1H, J = 8.1 Hz), 7.51-7.62 (m, 3H), 7.94-7.96 (m, 2H), 8.91 (brs, 1H), 9.45 (brs, 1H).^{13}C NMR (75 MHz, $CDCl_3$) δ: 24.1, 27.1, 53.7, 116.5 (q, J_{C-F} = 3.8 Hz), 120.8, 124.4 (q, J_{C-F} = 269.9 Hz), 127.1, 129.2, 132.2, 134.0, 134.8, 145.8, 165.1. HRMS ($M+H^+$): Calculated for $C_{19}H_{20}F_3N_2O$, 349.1528; found: 349.1451.

4.1.4.19 N-(2-(cyclohexylamino)-5-(trifluoromethyl)phenyl)benzamide (**32**)

The compound was obtained as a white solid in 55% yield after purification by column chromatography eluted with hexane-ethyl acetate (5:1 v/v). TLC R_f = 0.43 (hexane-ethyl acetate 5:1 v/v). mp 157.5-158.8 ºC. IR (ATR, cm^{-1}) $\bar{\nu}_{max}$:3396, 3214, 3058, 2935, 2862, 1636, 1613, 1552, 1334, 1243, 1213, 1161, 1105, 1073, 880, 812, 707, 624. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.13-2.04 (m, 10H), 3.27-3.33 (m, 1H), 4.10-4.22 (brs, 1H), 6.79 (d, 1H, J = 8.7 Hz), 7.38 (dd, 1H, J = 8.6 Hz and$J$ = 1.4 Hz), 7.46-7.60 (m, 4H), 7.76 (brs, 1H), 7.89 (d, 2H, J = 7.5 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 25.0, 25.9, 33.2, 51.8, 112.7, 118.3 (q, J_{C-F}= 32.7 Hz), 122.9, 125.1 (q, J_{C-F} = 3.6 Hz), 127.5, 129.0, 132.5, 133.9, 145.0, 166.5. The signal of the carbono of the CF_3 group was of low intensity and it was not noticed in the spectrum. HRMS ($M+H^+$): Calculated for $C_{20}H_{22}F_3N_2O$, 363.1684; found: 363.1613.

4.1.4.20 N-(2-(pyrrolidin-1-yl)-5-(trifluoromethyl)phenyl)benzamide (**33**)

The compound was obtained in 80% yield as a white solid after purification by silica gel column chromatography eluted with hexane - ethyl acetate (5:1 v/v). TLC R_f = 0.30 (hexane-ethyl acetate 5:1 v/v). mp 122.2 - 122.8 ^{o}C.IR (ATR, cm^{-1}) $\bar{\nu}_{max}$: 3242, 2986, 2948, 2870, 1637, 1616, 1578, 1519, 1488, 1366, 1331, 1266, 1149, 1098, 1082, 874, 799, 695, 656. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.96 - 2.01 (m, 4H), 3.13 - 3.17 (t, 4H, J = 6.3 Hz), 7.15 (d, 1H, J = 8.4 Hz), 7.34 (dd, 1H, J = 8.4 Hz and J = 1.5 Hz), 7.49 - 7.61 (m, 3H), 7.89 - 7.91 (m, 2H), 8.51 (brs,1H), 8.70 (brs, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 24.9, 52.0, 118.5, 119.6 (q, J_{C-F} = 3.8 Hz), 122.0 (q, J_{C-F} = 3.9 Hz), 124.5 (q, J_{C-F} = 269.9 Hz), 124.8 (q, J_{C-F} = 32.5 Hz), 127.2, 129.2, 131.1, 132.2, 134.7, 144.6, 165.3. HRMS ($M+H^+$): Calculated for $C_{18}H_{18}F_3N_2O$, 335.1371; found: 335.1277.

4.1.4.21 N-(2-(diethylamino)-5-(trifluoromethyl)phenyl)benzamide (**34**)

The compound was obtained in 84% yield as a white solid after purification by silica gel column chromatography eluted with hexane-ethyl acetate (5:1 v/v). TLC R_f = 0.65 (hexane - ethyl acetate 5:1 v/v). mp 65.1 - 66.0 ºC. IR (ATR, cm^{-1}) $\bar{\nu}_{max}$: 3334, 2975, 2932, 2858, 1678, 1586, 1534, 1483, 1440, 1336, 1247, 1167, 1150, 923, 828, 716. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.00 (t, 6H, J = 7.2 Hz), 3.03 (q, 4H, J = 7.2 Hz), 7.30 (d, 1H, J = 8.4 Hz), 7,36 (dd, 1H, J = 8.4 Hz and J = 1.5 Hz), 7.50 - 7.61(m, 3H), 7.92 (dd, 2H, J = 8.1 Hz and J = 1.5 Hz), 8.97 (brs, 1H), 9.81 (brs, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 13.0, 49.3, 116.2 (q, J_{C-F} = 3.9 Hz), 120.5 (q, J_{C-F} = 3.8 Hz), 123.4, 124.3 (q, J_{C-F} = 270.7 Hz), 128.1 (q, J_{C-F} = 32.3 Hz), 127.2, 129.1, 132.2, 134.9, 136.9, 142.5, 165.1. HRMS ($M+H^+$): Calculated for $C_{18}H_{20}F_3N_2O$, 337.1528; found: 337.1449.

4.1.4.22 N-(2-morpholino-5-(trifluoromethyl)phenyl)benzamide (**35**)

The compound was obtained as a white solid in 78% yield after recrystallization with acetone. TLC R_f = 0.18 (hexane-acetate 5:1 v/v). mp 137.3 - 138.5 ºC. IR (ATR, cm^{-1}) $\bar{\nu}_{max}$: 3369, 2967, 2896, 2851, 1668, 1589, 1534, 1465, 1438, 1335, 1238, 1157,

637 1112, 1075, 1025, 937, 917, 897, 877, 821, 801, 707, 659. ^{1}H NMR (300 MHz, $CDCl_3$)
638 δ: 2.96 (t, 4H, *J* = 4.5 Hz), 3.92 (t, 4H, *J* = 4.5 Hz), 7.30 (d, 1H, *J* = 8.4 Hz), 7.38 (dd,
639 1H, *J* = 8.4 Hz and *J* = 1.5 Hz), 7.52 - 7.63 (m, 3H), 7.93 (dd, 2H, *J* = 8.1 Hz and *J* =
640 1.5 Hz), 8.91 (brs, 1H), 9.39 (brs, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 52.5, 67.8, 116.9
641 (q, J_{C-F} = 3.8 Hz), 121.0, 121.1 (q, J_{C-F} = 3.9 Hz), 124.2 (q, J_{C-F} = 270.5 Hz), 127.0,
642 128,2 (q, J_{C-F} = 32.5 Hz), 129.2, 132.5, 134.1, 134.6, 144.0, 165.0. HRMS ($M+H^+$):
643 Calculated for $C_{18}H_{18}F_3N_2O_2$, 351.1320; found: 351.1266.

644 *4.1.4.22 N-(2-(4-bromophenylamino)-5-(trifluoromethyl)phenyl)benzamide* (**36**)

645 The compound was obtained as a white solid in 58% yield. TLC R_f = 0.25
646 (hexane - ethyl acetate 5:1 v/v). mp 157.3 - 158.0 ºC. IR (ATR, cm^{-1}) $\bar{\nu}_{max}$: 3366, 2967,
647 2892, 2852, 1668, 1589, 1534, 1465, 1438, 1335, 1238, 1157, 1112, 937, 917, 897,
648 877, 821, 802, 707, 659. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 6.30 (brs, 1H), 6.83 (d, 2H, *J* =
649 8.7 Hz), 7.31 - 7.47 (m, 6H), 7.55 (t, *J* = 7.4 Hz, 1H), 7.75 (d, 2H, *J* = 8.7 Hz), 8,05 (brs,
650 1H), 8.22 (brs, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 113.9, 119.7, 120.9, 121.1 (q, J_{C-F} =
651 3.9 Hz), 123.3 (q, J_{C-F} = 3.7 Hz), 123.9 (q, J_{C-F} = 270.1 Hz), 125.1 (q, J_{C-F} = 33.0 Hz),
652 127.1, 128.9, 129.3, 132.4, 133.3, 138.6, 141.8, 166.3. HRMS ($M+H^+$): Calculated for
653 $C_{20}H_{15}BrF_3N_2O$, 435.0320; found: 435.0300.

654 *4.2 Biological assays*

655 *4.2.1 Cell culture*

656 Human leukemia cell lines HL60 (acute myelogenous leukemia - AML), Nalm6
657 (B-cell acute lymphoblastic leukemia – ALL-B), and Jurkat (T-cell acute lymphoblastic
658 leukemia - ALL-T) were kindly provided by Dr. Jose Andrés Yunes (Centro Infantil
659 Boldrini, Campinas, São Paulo, Brazil). Cell lines were grown in RPMI-1640 medium
660 (Sigma) supplemented with 10% (v/v) fetal bovine serum (FBS) (LGC Biotecnologia),
661 100 g/mL streptomycin, and 100 units/mL penicillin (Sigma) at pH 7.2 and 37 ºC under
662 5% CO_2 atmosphere. Peripheral blood mononuclear cells (PBMC) were isolated from

human-heparinized blood using Histopaque-1077 (Sigma) according to the manufacturer's protocol. The isolated lymphocytes were resuspended in complete RPMI-1640 medium supplemented with 10% FBS and stimulated with 1% (v/v) phytohemagglutinin (Gibco). The cells were counted using a Neubauer chamber for the following experiments.

4.2.2 Cell viability assay

HL60, Nalm6, and Jurkat cells ($7x10^4$ cells/well) and PBMC ($1x10^5$ cells/well) were seeded in 96-well plates. Each well contained 100 µL of complete RPMI medium and 100 µL of each compound solution at different concentration. The compounds were diluted in RPMI medium with 10% FBS and 0.4% DMSO (v/v, Sigma). After 48 h of culture, MTT (5 mg/mL, Sigma) was added to the wells. After 3 h at 37 ºC, the MTT solution was removed and it was added 100 µL/well of DMSO to solubilize the formazan. Absorbance was measured at 540 nm in a microplate reader (SpectraMax M5, Molecular Devices).

4.2.3 Drug combination studies

Cell viability of leukemia cells treated with a combination of compounds **24**, **30**, or **36** with vincristine was assessed by seeding $7x10^4$ Nalm6 cells in each well of a 96-well plate. The cells were then incubated with each compound (at concentrations corresponding to 25 and 50% of the IC_{50}), vincristine (0.5 or 1.0 nM, Sigma) or a combination of each compound and vincristine for 48 h. The cell viability was determined by MTT assay and CompuSyn software was used to calculate the combination index (CI) as previously described [26].

4.2.4 Apoptosis assay by flow citometry

Nalm6 cells were seeded on 96-well plate at density of $7x10^4$ cells per well and treated with compounds **24**, **30** and **36** [20 µM]. DMSO (0.4% v/v) was used as vehicle control. After treatments, cells were labeled by using Annexin V/FITC apoptosis

689 detection kit I (BD Biosciences) according to manufacturer's protocol. Then the cell
690 samples were analyzed by flow cytometry (FACS Verse, BD Bioscience).

691 *4.2.5 Autophagy detection with acridine orange staining*

692 Nalm6 cells were seeded on 96-well plate at density of $7x10^4$ cells per well and
693 treated with compounds **24**, **30** and **36** [20 µM] or DMSO (0.4% v/v). After, cells were
694 washed with phosphate-buffered saline (PBS), suspended in PBS and stained by
695 acridine orange (1 µM, Sigma) at 37 ºC for 15 min; then the cells were washed with
696 PBS and resuspended in 0.5 mL of PBS. For visual examination of autophagosomes,
697 cells were analyzed under a fluorescence microscope Evos FL (Life technologies).

698 *4.2.6 Cell proliferation assay*

699 Proliferation assays were performed in 96-well plates containing $1x10^4$ Nalm6
700 cells per well or $1.5x10^4$ HL60 cells per well. The compounds **24**, **30**, and **36** were
701 added at 20 µM and DMSO (0.4% v/v) were used as control. The effect of each
702 treatments on cell growth were determined by trypan blue (Invitrogen) dye exclusion.
703 After 24, 48, 72, and 96 h cells were loaded on a hemocytometer to obtain the viable
704 cell count.

705 *4.2.7 RT-PCR assay*

706 Nalm6 cells were exposed to 20 µM of compounds **24**, **30**, and **36** or SRPIN340
707 for 24 h. Cells treated with DMSO (0.4% v/v) were used as control. After incubation,
708 mRNA was extracted using Tri Reagent (Sigma) according to the manufacturer's
709 protocol. Samples were quantified by spectrophotometry (NanoDrop, Thermo
710 Scientific) and analyzed for integrity in 1% agarose gel. Afterwards, the RNA was used
711 for first-strand cDNA synthesis using the Super Script First-Strand kit (Invitrogen)
712 according to the manufacturer's protocol. Then, the cDNA was used to amplify each
713 fragment of interest by PCR using the GoTaq Green Master Mix (Promega) kit, and the

products were separated in 1% or 2% agarose gels. All primers used in these assays are listed in **Supplementary Table 1**.

4.2.8 Western blotting assay

Nalm6 cells were treated with 20 µM of compounds **24**, **30**, and **36** or SRPIN340 for 24 h. After, cells were lysed in PBS containing 1% (v/v) NP40, 1 mM EDTA, 150 mM NaCl, protease and phosphatase inhibitors (Sigma), and 10 mM Tris (pH 7.4) at a concentration of $2x10^7$cells/mL in lysis buffer. Samples were incubated on ice for 10 minutes, briefly sonicated, and centrifuged for 10 minutes at 15000 x*g* to remove insoluble cellular debris. Proteins were resolved by SDS polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride (PVDF) membrane (GE Healthcare), blocked overnight in PBS containing 5% (w/v) skim milk powder, incubated for 2 h with primary antibody, and then incubated for 2 h with secondary antibody solutions. Primary antibodies used were mouse anti-SRPK1 (BD Biosciences), mouse anti-SRPK2 (BD Biosciences), rabbit anti-actin (Sigma) and mouse anti-phospho SR proteins mAb1H4 (Invitrogen). The last one is able to detect different phospho-SR proteins epitopes [4, 33]. The secondary antibodies used were anti-mouse peroxidase-conjugated (Sigma) and anti-rabbit peroxidase-conjugated (Sigma). Then, proteins were visualized using 3,3'-Diaminobenzidine tetrahydrochloride (Sigma) according to the manufacturer's protocol.

4.2.9 Statistical analysis

All numeric data were obtained from three independent experiments and are shown as means ± standard deviation. Analyses were performed using Microsoft Excel (Microsoft Office Software) and GraphPad Prism (GraphPad Software Inc.). Statistical analyses were done by one-way ANOVA followed by Dunnett's test. *$P < 0.05$ was considered significant.

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Captions

Fig. 1. SRPK inhibitors with biological activity.

Fig. 2. Effect of compounds **24**, **30**, and **36** over peripheral blood mononuclear cells (PBMC) stimulated with phytohemagglutinin (PHA). Cells were treated with 25 µM of each compound for 48 h. Cell viability was determined using MTT assay. Control treatment (vehicle) was considered 100% of viability. Data are shown as means ± standard deviation of triplicate experiments (*$P < 0.05$).

Fig. 3. Effect of compounds **24**, **30**, and **36**, in combination with vincristine, on the growth inhibition of Nalm6 cells. Cells were plated onto 96-well plates containing indicated concentrations of compound **24** (A), compound **30** (B), and compound **36** (C) or vincristine alone or in combinations with a fixed ratio for 48 h. The percentages of surviving cells as compared to controls, defined as 100% of viable cells, were determined by MTT assay. The combination index (CI) values were calculated using CompuSyn software according to the Chou–Talalay equation [26]. Synergistic effect is characterized by CI < 1.0, additive effect by CI close to 1.0 and antagonistic effect by CI > 1.0. Data are shown as means ± standard deviation of triplicate experiments.

Fig. 4. Effect of compounds **24**, **30,** and **36** on leukemia cell death. (A) Nalm6 cells were treated with 20 µM of each compound for 12 and 24 h. Cells treated with vehicle (DMSO) were used as control. Apoptosis/necrosis was evaluated using annexin-

V/FITC and PI labels. One representative experiment is shown. (B) The graphs show averaged percentage of apoptotic cells (annexin-V positive cells) of triplicate experiments. *P < 0.05. To assess the autophagossome induction (C), Nalm6 cells were treated with 20 µM of each compound or DMSO for 24 h. Subsequently, cells were stained with acridine orange and visualized under fluorescent microscopy. White arrows point to the autophagosomes. One representative experiment of three is shown.

Fig. 5. Effect of compounds **24**, **30,** and **36** on leukemia cell proliferation. (A) HL60 and (B) Nalm6 cells were treated with 20 µM of each compound. Cells treated with vehicle (DMSO) were used as control. Cell growth was determined with trypan blue exclusion at 0, 24, 48, 72, and 96 h after incubation (*P < 0.05).

Fig. 6. Effect of compounds **24**, **30**, and **36** in the intracellular activity of SRPKs**.** Nalm6 cells were treated with 20 µM of each compound for 24 h in order to investigate the effect on gene expression by RT-PCR assays (A) and SR protein phosphorylation pattern by Western blotting assays (B). Cells treated with vehicle (DMSO) or SRPIN340 [20 µM] were used as control. One representative experiment of three is shown for each analysis. (*) represent possible spliced isoforms and (**) represent the phosphorylated SRSF5 splicing factor.

Scheme 1. Nucleophilic aromatic substitution reactions between compound **1** and different amines involved in the preparation of compounds **2**-**7**.

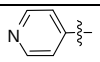
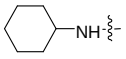
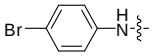
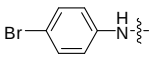
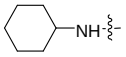
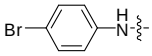
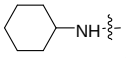
Scheme 2. Reduction of compounds **2**-**7** with $SnCl_2$/HCl.

Scheme 3. Final step involved in the preparation of SRPIN340 and compounds **15**-**36**.

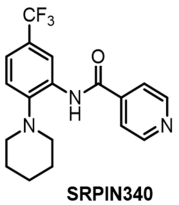
Table 1. Synthesized compounds and half-maximal inhibitory concentration (IC_{50}) values over leukemic cell lines. HL60 (AML), Jurkat (LLA-T) and Nalm6 (LLA-B) cells were treated with increasing concentrations (0 – 200 µM) of each compound for 48 h.

914 Cell viability was determined using the MTT assay. The IC_{50} values are expressed as

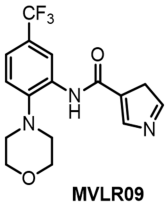
915 the means ± standard deviation of three independent experiments.

Compound	R^1	Ar	Yeld (%)	IC_{50}		
				HL60	Jurkat	Nalm6
SRPIN340			75	38.3±8.7	75.4±5.7	70.6±5.0
15			82	59.2±5.0	80.9±6.7	59.0±2.8
16			70	NA	NA	NA
17			85	89.7±12.8	NA	63.6±6.6
18			78	NA	NA	NA
19			81	NA	NA	51.9±0.8
20			78	NA	NA	NA
21			79	NA	NA	NA
22			59	84.1±6.0	88.4±11.9	NA
23			91	NA	NA	NA
24			37	14.2±0.9	20.6±4.0	35.7±1.0
25			65	NA	NA	NA
26			53	48.3±3.9	NA	52.3±3.7
27			73	NA	NA	NA
28			87	71.0±2.3	NA	63.2±2.0
29			74	NA	NA	NA
30			30	8.5±0.2	17.8±1.1	17.0±1.0
31			88	NA	NA	NA
32			55	34.9±1.7	NA	NA
33			80	NA	NA	NA
34			84	NA	NA	NA
35			78	NA	NA	NA
36			58	11.8±0.4	33.8±1.8	6.0±2.4

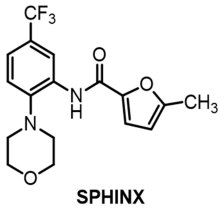
NA: Not active within the concentration range evaluated (0-200 µM); IC_{50} values expressed in µM; AML: acute myelogenous leukemia; ALL-T: T-cell acute lymphoblastic leukemia; ALL-B: B-cell acute lymphoblastic leukemia.



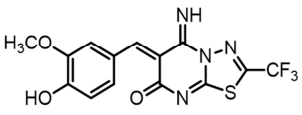
SRPIN340



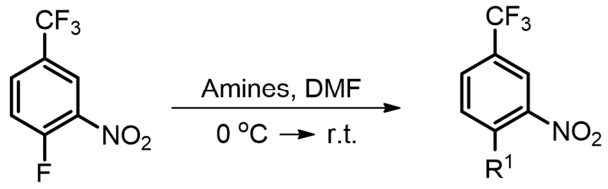
MVLR09



SPHINX

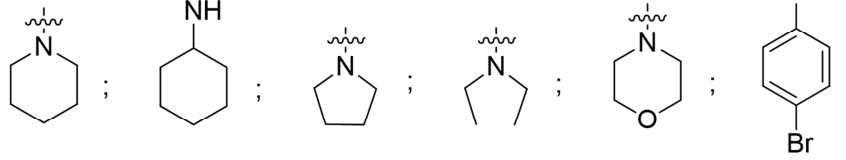


SRPIN803

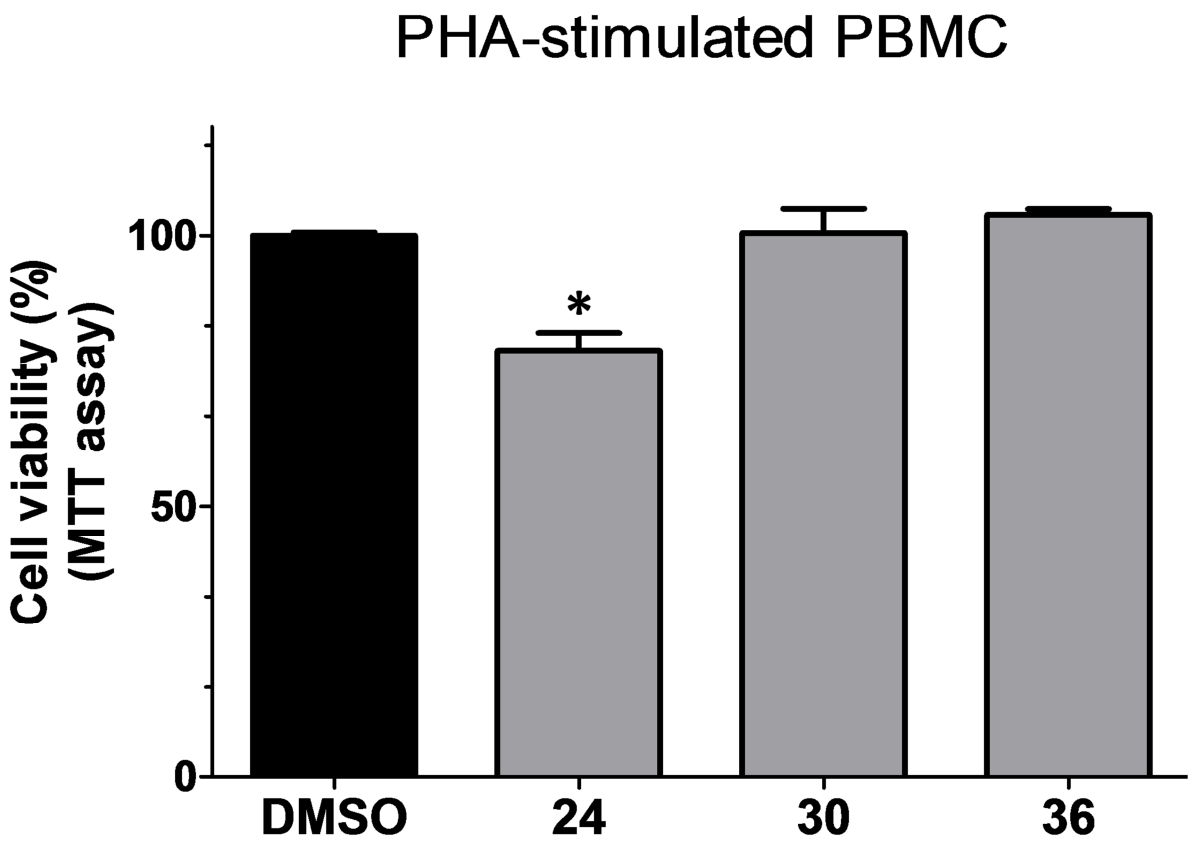


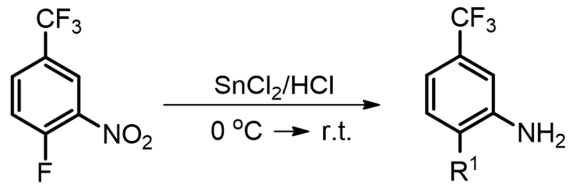
(**1**) (**2-7**)

Amines = piperidine, ciclohexylamine, pirrolidine, diethylamine, morpholine, 4-bromo aniline

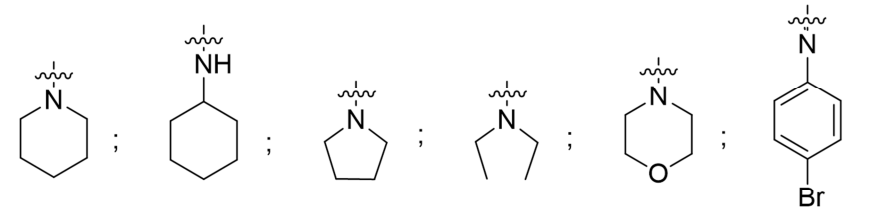
R^1 = 

(**2**, 91%) (**3**, 81%) (**4**, 97%) (**5**, 98%) (**6**, 97%) (**7**, 93%)

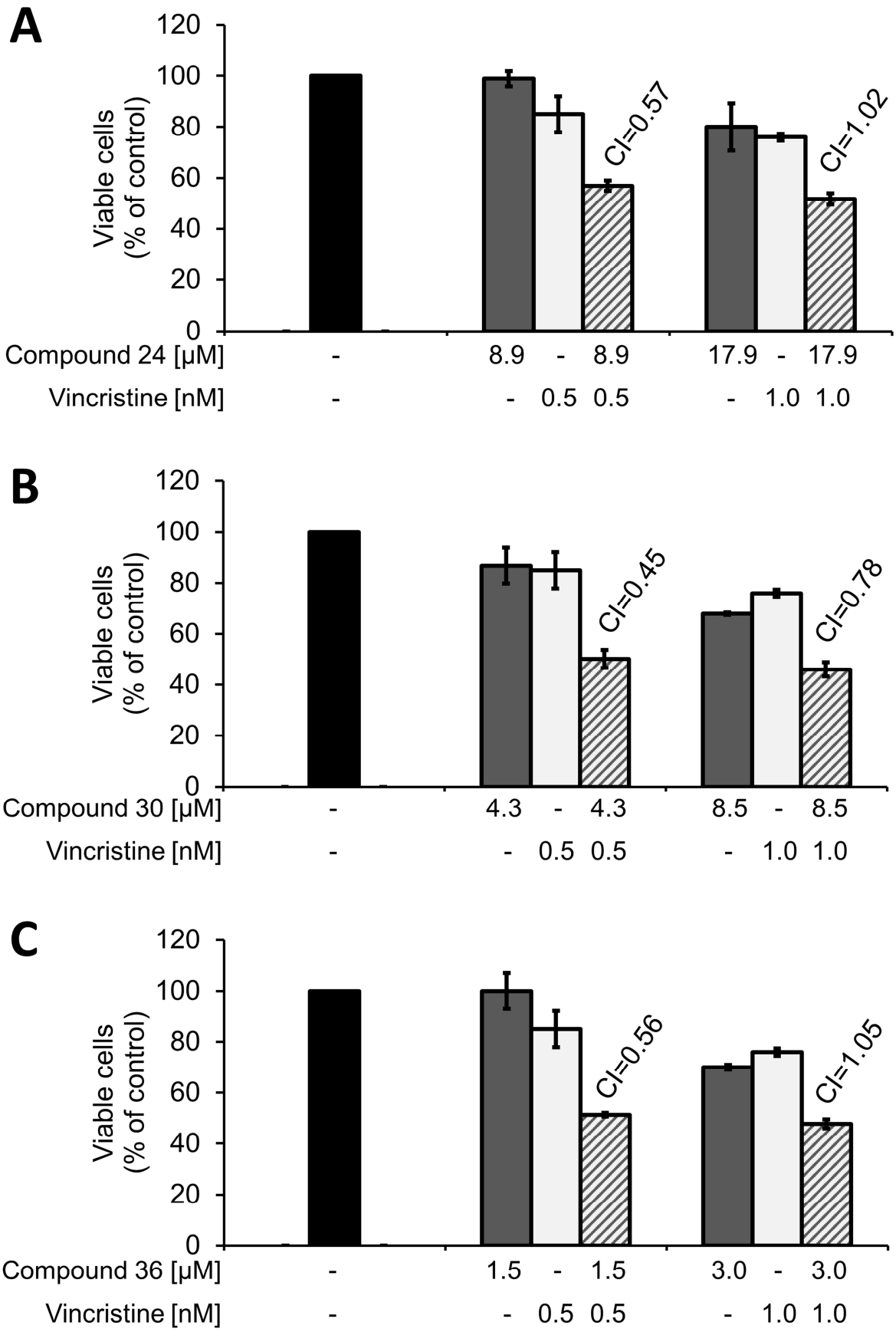


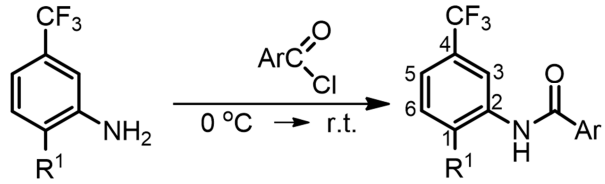


(**2-7**) (**8-13**)

R^1 =  ; ; ; ; ;

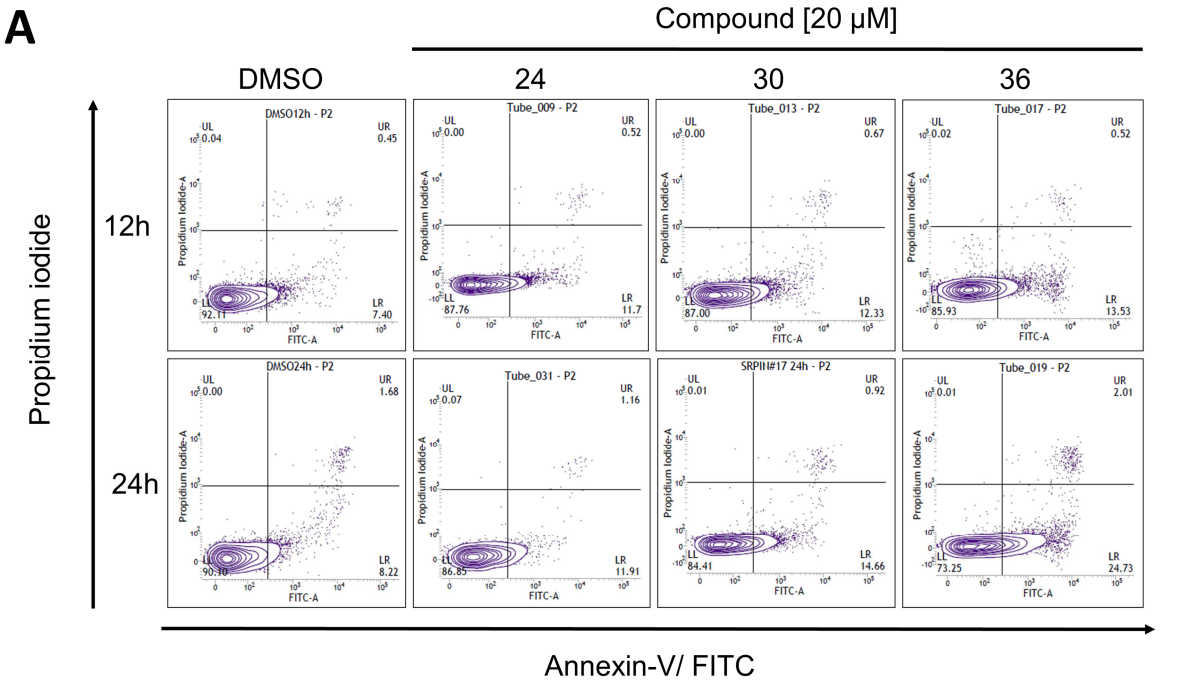
(**8**, 78%) (**9**, 56%) (**10**, 77%) (**11**, 67%) (**12**, 94%) (**13**, 79%)

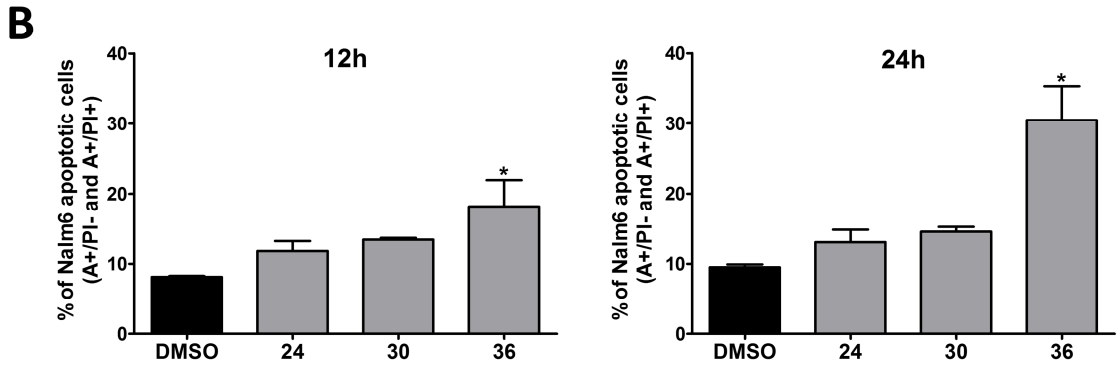

A
Viable cells (% of control)
120
100
80
60
40
20
0
CI=0.57
CI=1.02
Compound 24 [μM] - 8.9 - 8.9 17.9 - 17.9
Vincristine [nM] - - 0.5 0.5 - 1.0 1.0
B
Viable cells (% of control)
CI=0.45
CI=0.78
Compound 30 [μM] - 4.3 - 4.3 8.5 - 8.5
Vincristine [nM] - - 0.5 0.5 - 1.0 1.0
C
Viable cells (% of control)
CI=0.56
CI=1.05
Compound 36 [μM] - 1.5 - 1.5 3.0 - 3.0
Vincristine [nM] - - 0.5 0.5 - 1.0 1.0

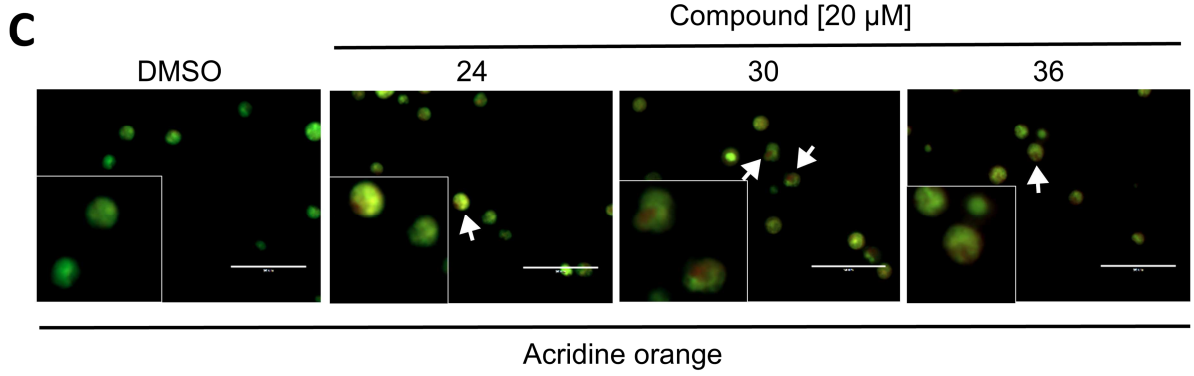


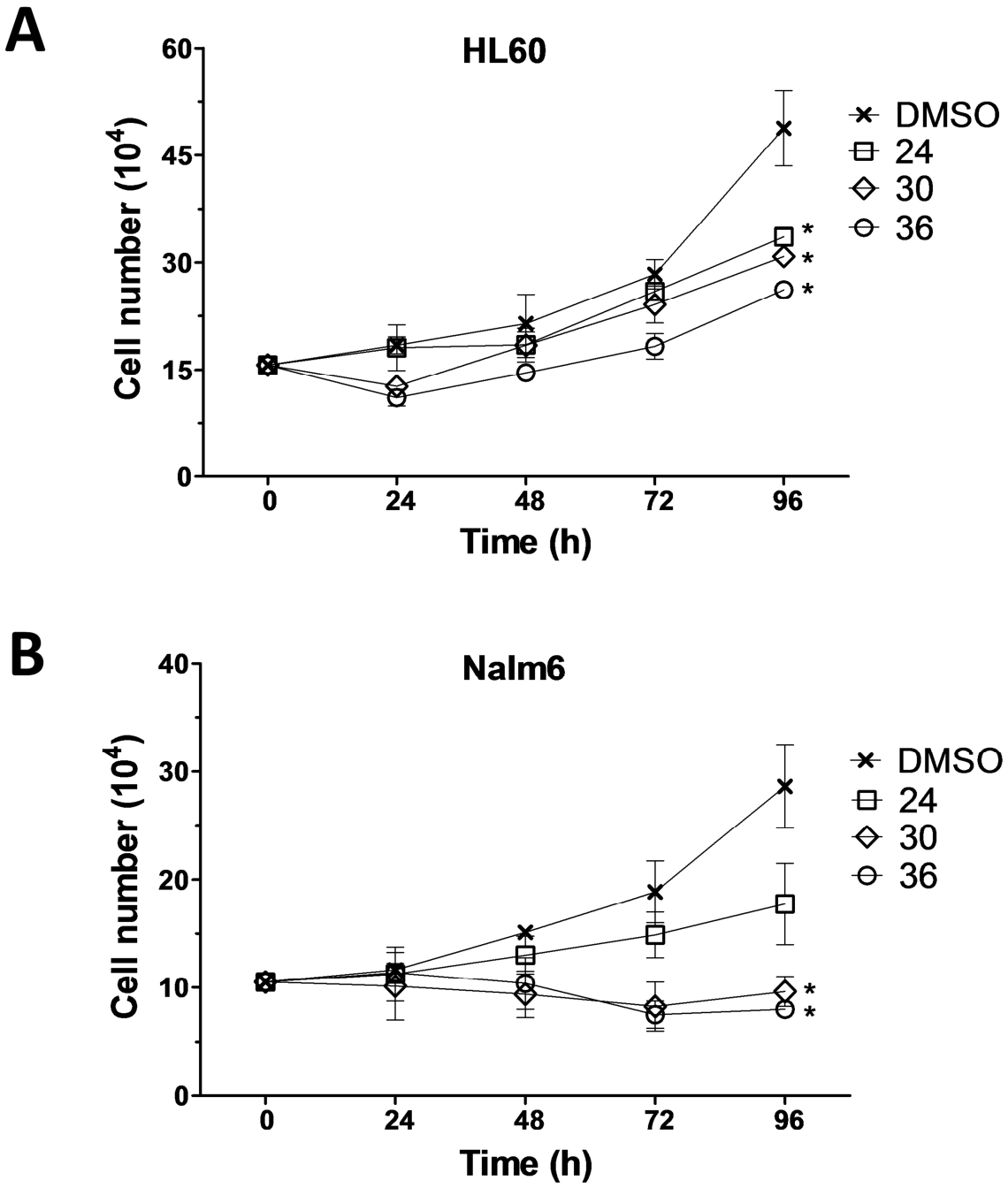
(**8-13**)

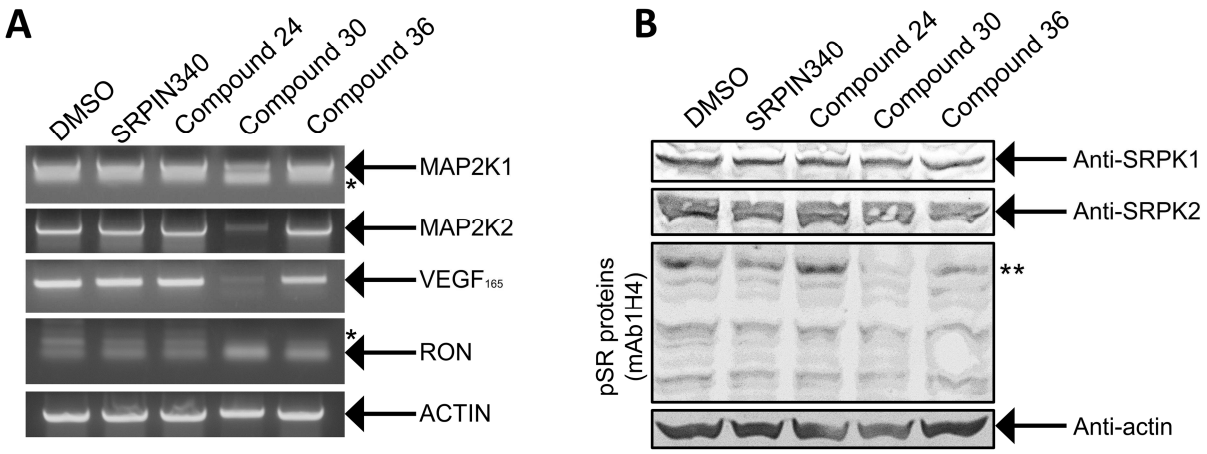
SRPIN340 and Compounds 15-36


A
Compound [20 μM]
DMSO
24
30
36
12h
24h
Propidium iodide
Annexin-V/ FITC
DMSO12h - P2
Tube_009 - P2
Tube_013 - P2
Tube_017 - P2
DMSO24h - P2
Tube_031 - P2
SRPIN#17 24h - P2
Tube_019 - P2


B
12h
24h
% of Nalm6 apoptotic cells (A+/PI- and A+/PI+)
DMSO
24
30
36


C
Compound [20 μM]
DMSO
24
30
36
Acridine orange


A
HL60
Cell number (10^4)
60
45
30
15
0
0
24
48
72
96
Time (h)
DMSO
24
30
36
*
*
*
B
Nalm6
Cell number (10^4)
40
30
20
10
0
0
24
48
72
96
Time (h)
DMSO
24
30
36
*
*



Highlights

- Trifluoromethyl arylamides were synthesized aiming at the inhibition of SRPKs.
- Substances with cytotoxic effect against leukemia cell lines were identified.
- The most active compounds induced apoptosis and autophagy in leukemia cells.
- The most active compounds presented synergistic effect with vincristine.
- Increased inhibition of SRPKs cellular activity was observed.