Antiproliferative effects of chalcones in the T-cell acute lymphoblastic leukemia-derived cells: role of PKCβ

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Abstract

A series of twenty chalcone derivatives was synthetized and their anti-proliferative activity was tested against the human T-cell acute lymphoblastic leukemia-derived cell line (CCRF-CEM). Based on the structural features of the most active compounds a new library of chalcone derivatives, according to SAR design, was synthetized and their antiproliferative activity was tested on the same cancer cell line. Four of them (compounds **3**, **4**, **8**, **28**), based on lower IC_{50} values (between 6.1 and 8.9 μM), were selected for further investigation regarding the modulation of the protein expression of receptor for activated C kinase (RACK1), PKCα and PKCβ, and their action at cell cycle level. Cell cycle analysis indicated a block in G0/G1 phase for all four compounds, with a statistically significant decrease in the percentage of cells in S phase, with no indication of apoptosis (subG0/G1 phase). Compounds **4** and **8** showed a statistical significant reduction in the expression of PKCα and an increase in PKCβ, which together with the demonstration of an antiproliferative role of PKCβ as assessed by treating cells with a selective PKCβ activator, indicated that the antiproliferative effect observed is likely to be mediated through PKCβ induction.

1. Introduction

Chalcones are natural occurring compounds produced by plants, precursors of flavonoids, including in their structure two aromatic rings joined by an α,β-unsaturated carbonyl system (Figure 1). They have been exploited for wide application in pharmacological area, due to their diverse biological activities, such as antimicrobial, antibacterial, antifungal, anti-inflammatory, anti-nociceptive properties as well as antitumor activity observed in different types of cancer, including leukemia, non-small cell lung cancer, colon cancer, prostate cancer and breast cancer.[1, 2]

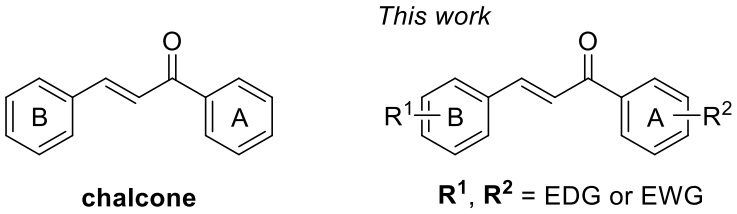


Figure 1. General structure of chalcone and synthesized derivatives.

Cancer is matter of concern in medicinal chemistry, and an increasing burden to the population, where the deaths from cancer worldwide are projected to reach over 13 million in 2030 (World Health Organization, https://www.who.int/cancer/resources/keyfacts/en/ visited Jan 8, 2020). The new generations of anticancer drugs are designed to target signals that promote or regulate the cell cycle, growth factors or their receptors, pathways affecting DNA repair and cell death rather than targeting directly DNA synthesis.[2] Many different cellular targets of chalcones have been highlights in cancer cells, as repressing Aurora Kinase A gene (AURKA) in breast cancer[3] or reveling their potential reversal activity against P-gp-mediated MDR, involved in resistance mechanism,[4, 5] (as inducing poly-ADP-ribose polymerase cleavage and stabilizing p53 in a dose-dependent manner in colorectal carcinoma.[6] Recently, chalcone derivatives were shown to inhibit Notch signaling in CCRF-CEM.[7, 8]

T-Acute lymphoblastic leukemia (ALL) is one of the most aggressive blood cancer, which account for approximately 15% of pediatric and 25% of adult acute lymphoblastic leukemia, in most cases curable.[9] However, chemotherapy resistance occurred in patients with T-ALL, leading to treatment failure and early relapse, and the causes of the occurrence of this resistance are not completely elucidated.[10] In a recent study, it has been shown that the overexpression of the receptor of activated C kinase 1 (RACK1) was responsible for chemotherapy resistance of T-cell acute lymphoblastic leukemia (T-ALL) increasing the activity of isoform α of protein kinase C (PKCα), which lead to a reduction in the level of Apaf-1, caspase 3 and FEM1b.[11]

RACK1 has been identified in the early nineties and so named because of its interaction with Protein Kinase C (PKC).[12] RACK1 is now recognized as a multiple target scaffolding protein involved in several key biological events, including development, immune response, neuronal activity, and cancer.[13, 14] Due to its plethora of interaction proteins, RACK1 controls essential cellular processes, such as transcription and translation, cell proliferation and growth as well as cell spreading and cell-cell interactions.[13] Regarding PKC, RACK1 could serve as a receptor for activated PKCβ and other PKC isoforms, including PKCδ and PKCμ. The binding of RACK1 to PKC leads to an increase in kinase activity, and to shuttle activated PKC to its correct cellular location. As early recognized, PKC activity and/or modulation of its isoenzyme expression has been implicated in the regulation, both increase and decrease, of malignant cell proliferation, apoptosis, tumor invasiveness, and resistance phenotype.[15-18] It is currently recognized that PKC isoforms have a complex not univocal role in cancer, meaning that they can have both detrimental or protective roles depending on the type of tumor considered, and therefore the use of PKC modulators must be done with caution taking into account the specific role of the different isoforms in the different tumors. Many PKC inhibitors have indeed entered clinical trials but has so far yielded very limited success, and in light of the fact that more recent studies demonstrated that PKC can also function as tumor suppressors, future clinical efforts should focus on restoring, rather than inhibiting, PKC activity.[19] Similarly to PKC, also RACK1 aberrant expression, pro- or anti-oncogenic effects, and contribution to the various stages of cell migration and invasion has been described in various cancers.[14, 20, 21]

Continuing our research in the synthesis of anticancer compounds,[22-29] the aim of this work was the synthesis of chalcone derivatives containing electron-withdrawing or electron-donating substituents on both the aromatic rings, and the evaluation of their antiproliferative effects, and possible modulation of PKC and RACK1 expression in the human T-cell acute lymphoblastic leukemia-derived cell line CCRF-CEM.

2. Results and discussion

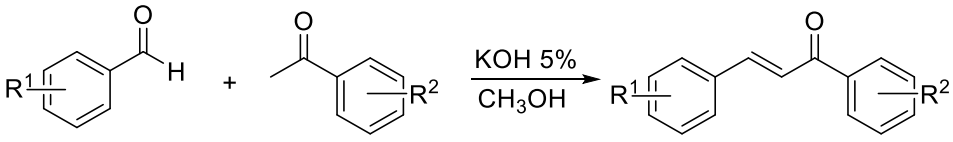
2.1. Chemistry

Chalcone derivatives were synthesized using the synthetic methodology previously described.[30, 31] Briefly, an aqueous solution of sodium hydroxide was added slowly to a methanol solution of the appropriate acetophenone. After the solution was cooled to room temperature, the appropriate benzaldehyde was added. The mixture was stirred at room temperature overnight to provide the corresponding chalcones in good yields (Table 1 and Table 2). The synthesis of products is easy to achieve with good grade of purity. On the contrary, in our hands, and also searching the literature data, the chalcone derivative with a *p*-NO_2 group on ring A was not able to be synthesized. All the products were characterized by NMR analysis, in which the presence of the olefinic double bond protons have a coupling constant *J* of 15.6 Hz confirming the *trans* stereochemistry.

2.2. Biological activity

The antiproliferative effects of the first library of the chalcone derivates **1**–**20** was evaluated in CCRF-CEM cells, a T lymphoblasts (T-ALL) from a 4 yrs old female isolated in 1964. For this blood tumor cell line a duplication time of approximately 24 h has been reported.[32] Cells were treated for 72 h, allowing three cell cycles, with increasing non-cytotoxic concentrations of the synthetized chalcones (0.16, 0.8, 4, 20 and 100 μM) or DMSO as vehicle control. The absence of cytotoxicity was assessed by lactate dehydrogenase leakage in a preliminary experiment testing compounds at 100 μM (data not shown). After 72 h, cell numbers were counted at the Coulter Counter, and the IC_{50} values calculated by linear regression analysis of data (IC_{50} is the concentration resulting in 50% inhibition of cell proliferation) and the results are presented in Table 1.

Table 1. Effect of chalcone derivatives on CCRF-CEM cells proliferation.



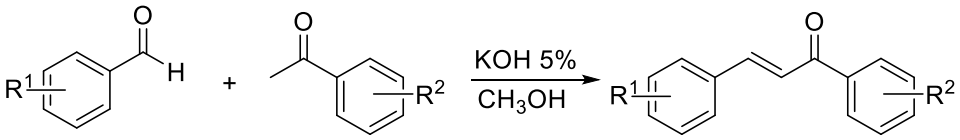
Compound	R^1	R^2	IC_{50} (μM)	Compound	R^1	R^2	IC_{50} (μM)
1	*o*-F	H	> 30	**11**	*p*-Me	*p*-Cl	> 30
2	*o*,*p*-Cl_2	H	> 30	**12**	*p*-Me	*p*-Ph	> 30
3	*m*-NO_2	H	8.9±1.2	**13**	*p*-Me	*p*-Me	> 30
4	*o*-CF_3	H	7.5±1.4	**14**	*p*-Me	*p*-OMe	> 30
5	*p*-OMe	H	7.9±1.2	**15**	*p*-Me	*p*-NO_2	> 30
6	2-naphthyl	H	> 30	**16**	*p*-OMe	*p*-Me	> 30
7	H	*p*-Me	> 30	**17**	*p*-OBn	*p*-Me	> 30
8	H	*m*-NO_2	6.1±1.1	**18**	*m*-NO_2	*p*-Me	> 30
9	H	*o*-OMe	6.8±1.1	**19**	*p*-OMe	*m*-NO_2	> 30
10	*p*-Me	*p*-Br	> 30	**20**	*p*-Cl	*p*-OMe	> 30

Cells were treated for 72 h with increasing non-cytotoxic concentrations of the synthetic chalcones. Cell proliferation was assessed by cell counting at the Coulter Counter. The IC_{50} (μM) values were calculated by linear regression analysis of data in three independent experiments. IC_{50} is reported as mean±SD.

Based on the results of the aforementioned screening and considering the antiproliferative activity of compounds **3**, **4**, **5**, **8** and **9**, which showed the lowest IC_{50} values (from 6.1 to 8.9 μM), a second library of chalcone derivatives was created, compounds **21** – **32**, based on the presence of the same electron-withdrawing or electron-donating substituents of compounds **3**, **4**, **5**, **8** and **9** on the aromatic rings. This second library will elucidate the importance of the single substituent in the antiproliferative activity based on structure-activity relationships. The antiproliferative activity of the new library against CCRF-CEM cells is presented in Table 2. The partition coefficient,

calculated for all the new compounds and for **3**, **4**, **5**, **8** and **9** showed good values for almost all of them.

Table 2. Effect of chalcone derivatives on CCRF-CEM cells proliferation.



Compound	R^1	R^2	IC_{50} (μM)	LogP	Compound	R^1	R^2	IC_{50} (μM)	LogP
21	*o*-NO_2	H	44.7±22.9	4.33	**27**	H	*o*-NO_2	14.7±0.5	3.41
3	*m*-NO_2	H	8.9±1.2	4.57	**8**	H	*m*-NO_2	6.1±1.1	5.19
22	*p*-NO_2	H	26.2±20.1	3.82	**28**	H	*o*-CF_3	11.4±2.2	4.87
4	*o*-CF_3	H	7.5±1.4	6.29	**29**	H	*m*-CF_3	14.8±3.2	5.9
23	*m*-CF_3	H	14.0±2.2	6.48	**30**	H	*p*-CF_3	49.5±21.0	5.7
24	*p*-CF_3	H	15.3±1.2	5.69	**9**	H	*o*-OMe	6.8± 1.1	4.58
25	*o*-OMe	H	13.7±1.7	4.48	**31**	H	*m*-OMe	14.0±3.7	5.26
26	*m*-OMe	H	27.1±1.8	5.03	**32**	H	*p*-OMe	20.4±9.4	3.99
5	*p*-OMe	H	7.9±1.2	4.51					

Cells were treated for 72 h with increasing non-cytotoxic concentrations of the synthetic chalcones. Cell proliferation was assessed by cell counting at the Coulter Counter. The IC_{50} (μM) values were calculated by linear regression analysis of data in three independent experiments. IC_{50} is reported as mean±SD.

The new SAR experiments showed that for the lower IC_{50} values the presence of the NO_2 on the *meta* position, CF_3 on the *ortho* position and the OMe group on the *para* position (compounds **3**, **4** and **5** respectively) as R^1 substituents and the NO_2 on the *meta* position , the CF_3 on the *ortho* position and the OMe group on the *ortho* position (compounds **8**, **28** and **9** respectively) as R^2 substituents and mandatory for the highest inhibition.

From the new library we considered chalcones **3**, **4** and **8**, **28** respectively which contain the same substituents with resonance or inductive electron-withdrawing effect at the same positions but on the opposite aromatic rings. These compounds were chosen for further analyses with the aim to investigate the mechanism underlying their antiproliferative effects.

Next, we investigated the effect of the selected compounds on the cell cycle. Cell cycle was evaluated by flow cytometry, assessing cellular DNA content following cell staining with propidium iodide and deconvolution of the cellular DNA content frequency histograms,[33] which allow to investigate distribution of cells in three major phases of the cycle (G0/G1 vs S vs G2/M) and makes it possible to detect apoptotic cells with fractional DNA content (pre-G0). Results are presented in Figure 2. Consistent with the lack of cytotoxicity, no increase in % of pre-G0 cells was observed, (Figure 2A), while an arrest in G0/G1 was observed for all selected compounds, characterized by a statistically significant increase in the % of cells in G0/G1 phase (Figure 2B) and a concomitant reduction of the cells in the S phase (Figure 2C). Results of the cell cycle analysis are consistent with cytostatic effect, rather than induction of apoptosis.

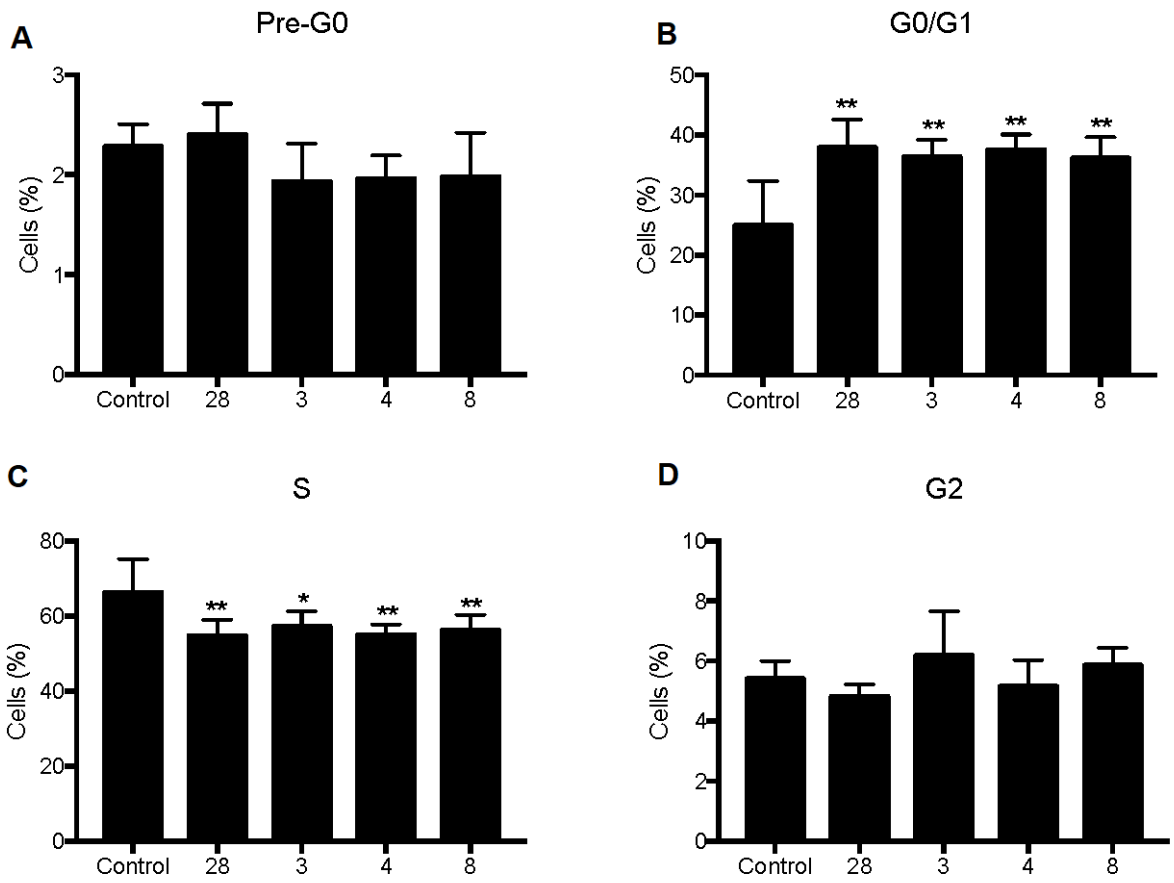


Figure 2. Cell cycle analysis following 72 h of treatment with the selected compounds in CCRF-CEM. Cells were treated for 72 with the selected compounds tested at the IC_{50} values or with DMSO as vehicle control. Cells were fixed and processed according to PI staining protocol listed in the Materials and Methods section. 30,000 cells were analyzed using flow cytometer. Cell cycle analysis calculated pre-G0, G0/G1, S and G2 phases from PI histograms. Each value represents mean±SD of three independent experiments. Statistical analysis was performed by ANOVA followed by Dunnett's multiple comparison test with *$p<0.05$ and **$p < 0.01$ versus control cells.

In CCRF-CEM the involvement of RACK1/PKC in chemotherapy resistance has been described.[11, 17] This together, with the role of RACK1/PKC in cancer,[14, 19] led us to investigate the effect of compounds **3**, **4**, **8** and **28** on the protein expression of PKCα, PKCβ and RACK1, and the role of PKCβ in cell proliferation by using the selective activator Pseudo RACK1. For protein expression,

compounds were tested at the IC_{50} values for 48 h. This early time point was chosen to see if changes in the expression levels occurred before the time point of 72 h used to assess the antiproliferative effect. The effect of Pseudo RACK1 on cell proliferation was evaluated at 72 h. Results are shown in Figure 3. A statistical significant decrease in PKCα expression was observed for compounds **4** and **8** (Figure 3A), while an increase in PKCβ expression was observed for compounds **3**, **4**, **8**, and no changes in RACK1 for all four compounds, indicating a selective action rather than an unspecific effect on protein synthesis. To understand the possible implication of the increased expression of PKCβ, Pseudo RACK1, a peptide which has been demonstrated both in vitro and in vivo to bind and activate PKC in the absence of PKC activators,[34] was used. Cells were treated for 72 h with increasing concentration of Pseudo RACK1, and cells counted at the end of the experiment (Figure 3D). A dose-dependent inhibition of cell proliferation was observed, suggesting that the increase in PKCβ induced by compounds **3**, **4**, **8** may probably explain the observed decrease in cell proliferation. The significance of the reduction of PKCα expression requires further studies, but consistent with the data published[11] a possible hypothesis could be a loss of chemotherapy resistance, which could be of extreme importance in the treatment of T-ALL.

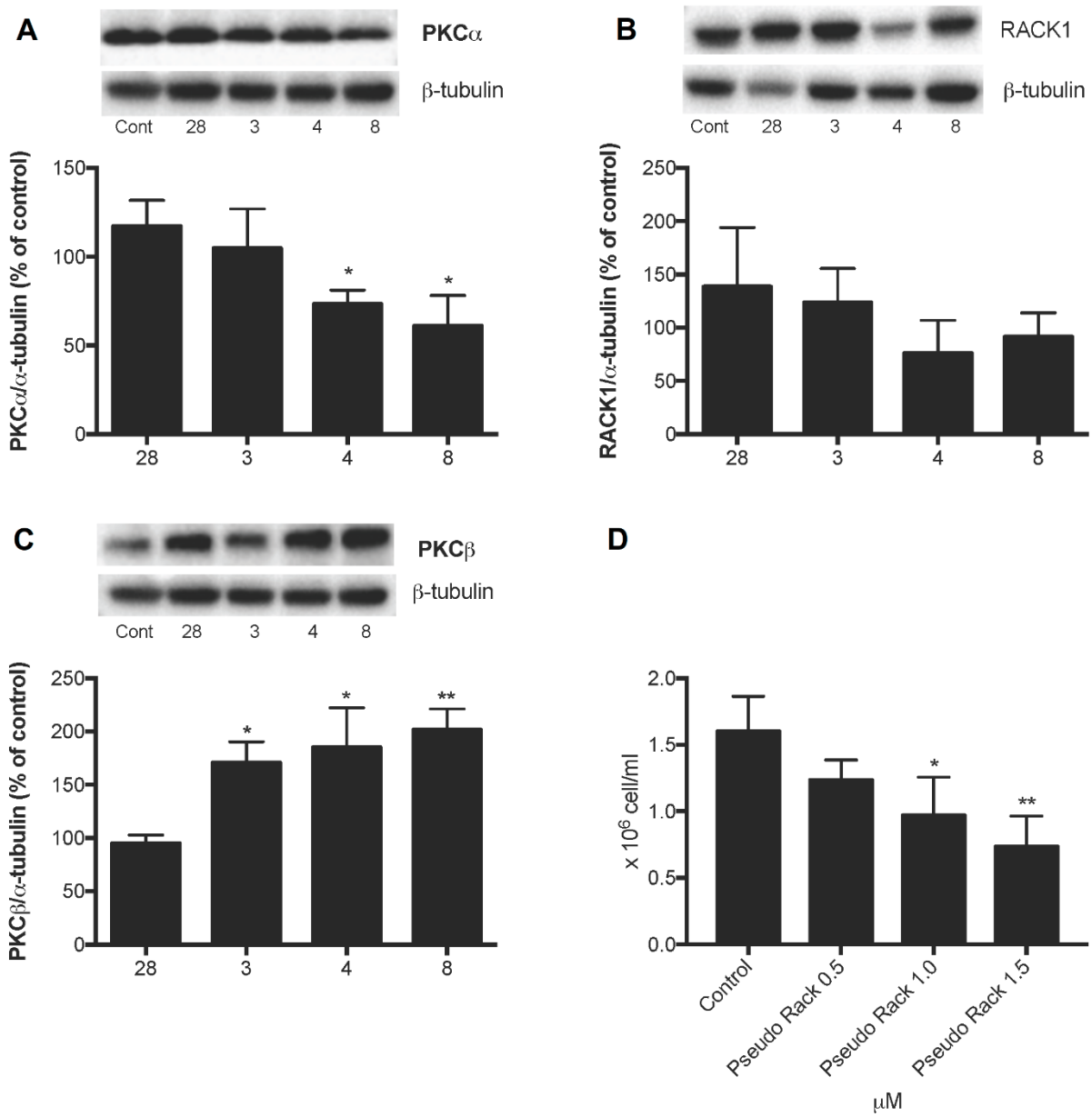


Figure 3. Effect of the selected chalcone derivatives on the α and β isoforms of PKC, RACK1 expression and antiproliferative effect of PKCβ activation in CCRF-CEM. A, B, C. Effect on PKCα (A), PKCβ (B) and RACK1 protein expression. Cells were treated for 48 h with the compounds **28**, **3**, **4**, **8** at the IC_{50} values or DMSO as vehicle control. β-tubulin expression was used to normalize expression. The image is a representative western blot. Each value represents the mean ± SD, n=3 independent experiments. Statistical analysis was performed with Dunnett's multiple comparison test with *$p<0.05$, ** $p<0.01$ versus control (Cont). D. Effect of PKCβ activation on CCRF-CEM proliferation. Cells were treated for 72 h with increasing concentrations of pseudo RACK1 (0.5-1.5

μM), a selective peptide activator of PKCβ. Cell proliferation was assessed by cell counting at the Coulter Counter. Each value represents the mean ± SD, n=3 independent experiments. Statistical analysis was performed with Dunnett's multiple comparison test with *$p<0.05$, ** $p<0.01$ versus control.

3. Conclusion

The preparation of two different libraries of chalcone derivatives, the first for an initial screening and the second for a structure-activity relationship identification, bearing electron-withdrawing or electron-donating substituents is described and the compounds were tested for their antiproliferative activity against CCRF-CEM cells. From the first screening, compounds **3**, **4**, **5**, **8** and **9**, which showed the highest antiproliferative activity were selected as lead compounds for the synthesis of the second library. After the second screening, chalcones **3**, **4**, **8** and **28** bearing electron-withdrawing substituents at the same positions but on the opposite aromatic rings, were chosen for further investigation. Cell cycle analysis of these compounds demonstrated a cytostatic effect, rather than induction of apoptosis. Moreover, regarding the protein expression of PKCa, PKCβ and RACK1, a significant decrease in PKCα expression was observed for compounds **4** and **8**, while an increase in PKCβ expression was detected for compounds **3**, **4** and **8**. Although, no changes in RACK1 for all four compounds was discovered suggesting a selective action rather than an effect on the protein synthesis. A dose-dependent inhibition of the cell proliferation with the peptide PseudoRACK1 suggested that the increase in PKCβ induced by compounds **3**, **4** and **8** could explain the decrease in cell proliferation. Even if further experiments are needed to better characterize the effect on PKC isoforms, the mechanisms underlying the modulatory effects observed, i.e. pre- or post-transcriptional effect, and their implication in term multidrug resistance compounds **4** and **8** have been identified as candidates for further investigation and development as additional therapeutic option in T-cell acute lymphoblastic leukemia.

4. Experimental section

4.1. General procedures

All reactions were monitored by TLC on silica gel, with detection by UV light (254 nm). ^{1}H NMR and ^{13}C NMR spectra were recorded with Varian Oxford 300 MHz spectrometer at 300 and 75 MHz respectively. MS analyses were performed by using a Thermo Finnigan (MA, USA) LCQ Advantage system MS spectrometer with an electrospray ionization source and an 'Ion Trap' mass analyzer. The MS spectra were obtained by direct infusion of a sample solution in MeOH under ionization, ESI positive.

4.2. General Procedure for the synthesis of α,β-unsaturated ketones

To a methanol solution (30 mL) of acetophenone (5.0 mmol) an aqueous solution of potassium hydroxide (5 %, 25 mL) was slowly added at 0°C. The appropriate benzaldehyde (6.0 mmol) was added and the new solution was stirred overnight at the room temperature. The resulting solution was evaporated *in vacuum*, EtOAc was added and the organic phase was washed with water and brine, dried with Na_2SO_4, filtered and evaporated. The desired product was obtained after purification by flash column chromatography.[30]

4.2.1 (*E*)-3-(2-Nitrophenyl)-1-phenylprop-2-en-1-one (21): ^{1}H NMR (300 MHz, $CDCl_3$) δ = 8.24-8.15 (m, 4H), 7.88 (d, *J*= 6.0 Hz, 1H), 7.84-7.82 (m, 1H), 7.76-7.71 (m, 1H), 7.56-7.47 (m, 4H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ = 188.13, 157.34, 148.30, 139.69, 136.71, 129.75, 129.61, 129.28, 128.80, 127.55, 127.42, 127.17, 126.24, 118.96 ppm. MS (ESI) for $C_{15}H_{11}NO_3$: m/z 254.37 $[M+H]^+$.

4.2.2. (*E*)-3-(3-Nitrophenyl)-1-phenylprop-2-en-1-one (3): ^{1}H NMR (300 MHz, $CDCl_3$) $_{\delta}$ = 8.49 (s, 1H), 8.23 (d, *J*=9.0 Hz, 1H), 8.03 (d, *J*= 8.4 Hz, 2H), 7.92 (d, *J*=9.0 Hz, 1H), 7.84-7.79 (m, 1H), 7.67-7.50 (m, 5H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ = 189.58, 141.56, 137.56, 136.65, 134.24, 133.26, 130.70, 130.01, 128.77, 128.56, 124.61, 122.33 ppm. MS (ESI) for $C_{15}H_{11}NO_3$: m/z 254.61 $[M+H]^+$.

4.2.3. (*E*)-3-(4-Nitrophenyl)-1-phenylprop-2-en-1-one (22): ^{1}H NMR (300 MHz, $CDCl_3$) δ = 8.28 (d, *J*=9.0 Hz, 2H), 8.03 (d, *J*=9.0 Hz, 2H), 7.80 (d, *J*=2.4 Hz, 2H), 7.78 (s, 1H), 7.67 (s, 1H), 7.63-7.51 (m, 3H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ = 188.85, 142.16, 137.33, 136.47, 133.98, 132.85, 130.96, 129.08, 128.52, 127.86, 123.88, 122.12 ppm. MS (ESI) for $C_{15}H_{11}NO_3$: m/z 254.48 $[M+H]^+$.

4.2.4. (*E*)-1-Phenyl-3-[2-(trifluoromethyl)phenyl]prop-2-en-1-one (4): ^{1}H NMR (300 MHz, $CDCl_3$) δ = 8.14 (dd, *J*=2.1 Hz, 15.6 Hz 1H), 8.03-7.95 (m, 2H), 7.82 (d, *J*=7.8 Hz, 1H), 7.73 (d, *J*=7.8 Hz, 1H), 7.63-7.58 (m, 2H), 7.54-7.48 (m, 3H), 7.43 (d, *J*=15.6 Hz, 1H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ = 190.28, 140.18, 140.16, 137.66, 134.03, 132.99, 132.06, 129.63, 129.42, 129.01, 128.64, 127.93, 126.62, 126.35, 126.28, 126.20, 126.13, 125.75, 122.12 ppm. MS (ESI) for $C_{16}H_{11}F_3O$: m/z 277.08 $[M+H]^+$.

4.2.5. (*E*)-1-Phenyl-3-[3-(trifluoromethyl)phenyl]prop-2-en-1-one (23): ^{1}H NMR (300 MHz, $CDCl_3$) δ = 8.04 (d, *J*=7.2 Hz, 2H), 7.89-7.79 (m, 3H), 7.68-7.50 (m, 6H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ = 189.96, 142.74, 137.82, 135.72, 133.03, 131.74, 131.54, 131.31, 129.48, 128.69, 128.52, 126.83, 126.78, 126.73, 126.69, 125.59, 124.73, 124.68, 124.63, 124.58, 123.73, 121.98 ppm. MS (ESI) for $C_{16}H_{11}F_3O$: m/z 277.44 $[M+H]^+$.

4.2.6. (*E*)-1-Phenyl-3-[3-(trifluoromethyl)phenyl]prop-2-en-1-one (24): ^{1}H NMR (300 MHz, $CDCl_3$) δ = 8.28 (d, *J*=9.0 Hz, 2H), 8.05-8.02 (m, 2H), 7.80-7.78 (m, 2H), 7.67 (s, 1H), 7.63-7.60 (m, 1H), 7.56-7.51 (m, 3H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ = 188.21, 149.98, 139.94, 136.62, 133.54, 131.89, 131.06, 128.14, 128.02, 127.92, 127.64, 126.89, 126.55, 126.13, 126.09, 125.99, 125.93, 125.01, 121.87 ppm. MS (ESI) for $C_{16}H_{11}F_3O$: m/z 277.36 $[M+H]^+$.

4.2.7. (*E*)-3-(2-Methoxyphenyl)-1-phenylprop-2-en-1-one (25): ^{1}H NMR (300 MHz, $CDCl_3$) δ =8.10 (d, *J*=15.9 Hz, 1H), 7.90 (d, *J*=7.3 Hz, 2H), 7.83 (d, *J*=7.6 Hz, 1H), 7.75-7.68 (m, 1H), 7.55-7.48 (m, 2H), 7.41-7.31 (m, 3H), 7.23 (d, *J*=15.7 Hz, 1H), 3.86 (s, 3H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ = 188.55, 157.12, 143.56, 137.19, 137.16, 135.66, 131.86, 131.13, 129.56, 128.18, 121.99, 121.00, 115.91, 112.87, 54.93 ppm. MS (ESI) for $C_{16}H_{14}O_2$: m/z 239.41 $[M+H]^+$.

4.2.8. (*E*)-3-(3-Methoxyphenyl)-1-phenylprop-2-en-1-one (26): ^{1}H NMR (300 MHz, $CDCl_3$) δ =8.03 (d, *J*=6.9 Hz, 2H), 7.78 (d, *J*=15.9 Hz, 1H), 7.54-7.46 (m, 4H), 7.35-7.30 (m, 1H), 7.25-7.23 (m, 1H), 7.16 (t, *J*=1.5 Hz, 1H), 6.98-6.94 (m, 1H), 3.84 (s, 3H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ = 190.47, 159.96, 144.70, 138.19, 136.27, 132.76, 129.93, 128.60, 128.49, 122.41, 121.07, 116.29, 113.47, 55.32 ppm. MS (ESI) for $C_{16}H_{14}O_2$: m/z 239.19 $[M+H]^+$.

4.2.9. (*E*)-3-(4-Methoxyphenyl)-1-phenylprop-2-en-1-one (5): ^{1}H NMR (300 MHz, $CDCl_3$) δ = 8.01 (d, *J*=8.1 Hz, 2H), 7.79 (d, *J*=15.9 Hz, 1H), 7.62-7.47 (m, 5H), 7.41 (d, *J*=15.6 Hz, 1H), 6.93 (d, *J*=8.7 Hz, 2H), 3.85 (s, 3H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ = 190.55, 161.68, 144.65, 138.53, 132.49, 130.18, 129.04, 128.52, 128.38, 128.00, 127.64, 119.85, 114.42, 113.61, 55.62 ppm. MS (ESI) for $C_{16}H_{14}O_2$: m/z 239.33 $[M+H]^+$.

4.2.10. (*E*)-1-(2-Nitrophenyl)-3-phenylprop-2-en-1-one (27): ^{1}H NMR (300 MHz, $CDCl_3$) δ = 8.18 (d, *J*=9.3 Hz, 1H), 7.76 (t, *J*=8.7 Hz, 1H), 7.65 (t, *J*=8.1 Hz, 1H), 7.52-7.48 (m, 3H), 7.41-7.37 (m, 3H), 7.25 (d, *J*=16.2 Hz, 1H), 7.00 (d, *J*=16.2 Hz, 1H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ = 192.83, 146.25, 136.31, 133.98, 133.95, 131.02, 130.56, 128.98, 128.79, 128.67, 128.52, 126.25, 124.51 ppm. MS (ESI) for $C_{15}H_{11}NO_3$: m/z 254.56 $[M+H]^+$.

4.2.11. (*E*)-1-(3-Nitrophenyl)-3-phenylprop-2-en-1-one (8): ^{1}H NMR (300 MHz, $CDCl_3$) δ = 8.83 (s, 1H), 8.38 (dd, *J*=8.1 Hz, 27.6 Hz, 2H), 7.89 (d, *J*=15.6 Hz, 1H), 7.74, 7.66-7.56 (m, 3H), 7.51 (d, *J*=15.6 Hz, 1H), 7.46-7.44 (m, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ = 187.97, 148.43, 146.73, 139.51, 134.31, 134.04, 131.17, 129.88, 129.08, 128.71, 127.01, 123.23, 120.68. MS (ESI) for $C_{15}H_{11}NO_3$: m/z 254.43 $[M+H]^+$.

4.2.12. (*E*)-3-Phenyl-1-[2-(trifluoromethyl)phenyl]prop-2-en-1-one (28): ^{1}H NMR (300 MHz, $CDCl_3$) δ= 7.97 (d, *J*= 8.9 Hz,1H), 7.58 (t, *J*= 7.4 Hz,1H), 7.55-7.53 (m, 1H), 7.50 (dd, *J*= 2.0 Hz, 7.5 Hz, 2H), 7.43 (d, *J*= 7.6 Hz, 1H), 7.40-7.35 (m, 3H), 7.31(d, *J*= 15.6 Hz, 1H), 7.02 (d, *J*= 15.4 Hz, 1H) ppm. ^{13}C NMR (75 MHz, DMSO) δ = 187.89, 146.12, 138.93, 138.51, 137.66, 135.05, 133.12, 131.67, 130.85, 130.03, 129.85, 129.66, 129.43, 129.12, 129.03, 128.88, 127.56, 125.13, 125.00, 122.02 ppm. MS (ESI) for $C_{16}H_{11}F_3O$: m/z 277.17 $[M+H]^+$.

4.2.13. (*E*)-3-Phenyl-1-[3-(trifluoromethyl)phenyl]prop-2-en-1-one (29): ^{1}H NMR (300 MHz, $CDCl_3$) δ = 8.26 (s, 1H), 8.20 (d, *J*= 7.8 Hz, 1H), 7.89-7.84 (m, 2H), 7.69-7.64 (m, 2H), 7.51 (d, *J*= 15.6 Hz, 1H), 7.45-7.44 (m, 2H), 7.23-7.14 (m, 2H) ppm. ^{13}C NMR (75 MHz, DMSO) δ = 188.89, 145.56, 139.33, 138.73, 137.36, 134.95, 132.93, 131.33, 130.55, 129.93, 129.90, 129.85, 129.58, 129.34, 128.70, 128.20, 127.06, 125.35, 125.30, 122.07 ppm. MS (ESI) for $C_{16}H_{11}F_3O$: m/z 277.31 $[M+H]^+$.

4.2.14. (*E*)-3-Phenyl-1-[4-(trifluoromethyl)phenyl]prop-2-en-1-one (30): ^{1}H NMR (300 MHz, $CDCl_3$) δ = 8.78-8.73 (m, 2H), 8.13 (d, *J*= 8.1 Hz, 2H), 7.54-7.40 (m, 3H), 7.25 (d, *J*= 7.8 Hz, 2H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ = 187.51, 143.82, 139.92, 137.72, 134.15, 131.89, 131.56, 127.94, 127.02, 127.00, 126.84, 125.94, 125.65, 125.82, 125.01, 124.99, 124.83, 124.06, 122.12 ppm. MS (ESI) for $C_{16}H_{11}F_3O$: m/z 277.54 $[M+H]^+$.

4.2.15. (*E*)-1-(2-Methoxyphenyl)-3-phenylprop-2-en-1-one (9): ^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.82 (d, *J*= 15.5 Hz, 1H), 7.65 (dd, *J*= 2.0 Hz, 7.5 Hz, 1H), 7.58–7.55 (m, 2H), 7.48–7.44 (m, 1H), 7.38–7.33 (m, 4H), 7.04-7.01 (m, 1H) 6.98–6.93 (m, 1H), 3.86 (s, 3H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ = 189.43, 156.97, 143.58, 138.18, 137.65, 136.44, 131.99, 131.76, 129.88, 128.10, 122.14, 121.14, 116.27, 112.76, 55.32 ppm. MS (ESI) for $C_{16}H_{14}O_2$: m/z 239.34 $[M+H]^+$

4.2.16. (*E*)-1-(3-Methoxyphenyl)-3-phenylprop-2-en-1-one (31): ^{1}H NMR (300 MHz, $CDCl_3$) δ = 8.03 (d, *J*= 15.6 Hz, 1H), 7.65–7.62 (m, 2H), 7.59 (dd, *J*= 1.8 Hz, 7.5 Hz, 1H), 7.56–7.54 (m, 1H), 7.52 (d, *J*= 15.6 Hz, 1H), 7.42–7.38 (m, 4H), 7.14 (dd, *J*= 3.0 Hz, 8.4 Hz), 3.85 (s, 3H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ = 189.72, 159.88, 143.87, 138.02, 136.83, 133.07, 129.98, 128.73, 128.05, 123.41, 122.14, 116.55, 114.71, 54.97. MS (ESI) for $C_{16}H_{14}O_2$: m/z 239.45 $[M+H]^+$.

4.2.17. (*E*)-1-(4-Methoxyphenyl)-3-phenylprop-2-en-1-one (32): ^{1}H NMR (300 MHz, $CDCl_3$) δ = 8.05–8.02 (m, 2H), 7.78 (d, *J*= 15.6 Hz, 1H), 7.65–7.61 (m, 2H), 7.53(d, *J*= 15.6 Hz, 1H), 7.44-7.39 (m, 3H), 6.98–6.96 (m, 2H), 3.83 (s, 3H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ = 189.12, 162.73, 145.87, 139.77, 133.12, 131.85, 129.65, 129.01, 128.77, 128.45, 127.93, 118.85, 114.32, 113.87, 54.69. MS (ESI) for $C_{16}H_{14}O_2$: m/z 239.17 $[M+H]^+$.

4.3. Log P_{ow} determination

RP-HPLC analysis were performed to correlate the hydrophobicity of the compounds with their retention time. The chromatograms were registered using Partisil C18-ODS reversed-phase HPLC column at 25 °C and water/acetonitrile (50:50) as mobile phase with KI as internal standard (flow rate: 1 mL/min; λ= 254 nm). The calibration curve was realized in comparison with reference compounds chosen in OECD guideline TG 107 (OECD TG 107).[35-37]

4.4. Biology

4.4.1. Cells

For all experiments the CCRF-CEM cell line was used (CEM/C1, ATCC CRL-2265^{TM}, gift form Luca Mazzarella, European Institute of Oncology, Department of Experimental Oncology, Milan, Italy). Cells were seeded at a density of $6x10^5$ cells/ml in RPMI 1640 containing 2 mM l-glutamine, 0.1 mg/ml streptomycin, 100 IU/ml penicillin and 0.1% gentamycin, supplemented with 10% heated-inactivated fetal calf serum (media) and cultured at 37°C in 5% CO_2 for 48 h for western blot analysis (3 ml per sample) and for 72 h for cell proliferation or cell cycle analysis (1 ml per sample). Cell culture media and all supplements were from Sigma-Aldrich Co. (St. Louis, Mo, USA). 50 mM stock solutions were prepared for each compound in dimethyl sulfoxide (DMSO). DMSO was used as vehicle control in all experiments (0.1% final concentration in culture medium). Pseudo RACK1 activator of protein kinase Cβ (pseudo RACK) was obtained from Tocris Bioscience (Bristol, UK). The pseudosubstrate consists of peptide derived from the C2 domain of PKCβ linked by a disulfide bridge to the Antennapedia domain vector peptide, which ensures rapid and effective uptake of the activator peptide. Once inside the cell, the disulfide bonds were subjected to reduction in the cytoplasm leading to release of the activator peptide.

4.4.2. Cell proliferation

Preliminary experiments were conducted to assess cell viability, $6x10^5$ cells/ml were treated with 100 μM of each compounds for 72 h. Cell viability was evaluated by assessing lactate dehydrogenase leakage using a commercially available kit (Takara Bio Inc., Japan), no cytotoxicity was observed (data not shown). Cells were seeded at a density of $6x10^5$ cells/ml and incubated for 72 h with five concentrations of the compounds (maximum concentration tested 100 μM, and four 1:5 dilutions) or DMSO as vehicle control. After incubation, cells were counted at the Coulter Counter (Coulter Electronics, Ltd., Luton, UK). From the dose response curve, the IC_{50} values (IC_{50} = effective chemical concentration required to inhibit reduce to 50% cell proliferation compared to vehicle exposed cultures) for each compound was calculated by linear regression analysis of data.

To assess the role of PKCβ on cell proliferation, $6x10^5$ cells/ml were treated with increasing concentrations of Pseudo RACK for 72 h. After incubation, cells were counted at the Coulter Counter, results are expressed as number of cells x 10^6/ml.

4.4.3. Cell cycle analysis by DNA content (propidium iodide, PI)

After 72 h incubation, cells were centrifuged for 5 min at 1200 rpm at 5°C, culture media was discarded, cells washed with PBS, fixed overnight at -20°C in 70% ethanol in PBS. Cells were then centrifuged for 5 min at 1200 rpm at 5°C, washed once with cold PBS, and resuspended in 1 ml of PI/Triton X-100 staining solution. Samples were incubated at 30 minutes at 25°C protected from light, and acquired using a FACSCalibur flow cytometer and data were quantified using CellQuest software (Becton Dickinson). 30.000 events were analyzed to determine cellular distribution in different phases of the cell cycle. Results are expressed as % of cells.[38]

4.4.4. Western blot analysis

For western blot analysis, after 48 h incubation, cells were centrifuged for 5 min at 1200 rpm at 5°C, culture media was discarded, cells washed with PBS. The supernatant was discarded, and cells lysed in 100 μl of homogenization buffer (50 mM TRIS, 150 mM NaCl, 5 mM EDTA pH 7.5, 0.5%

Triton X-100, 50 μM PMSF, 2 μg/mL aprotinin, 1 μg/mL pepstatin and 1 μg/mL leupeptin). 100 μl of sample buffer 2X (125 Mm Tris HCl pH 6.8, 4% SDS, 20% glycerol, bromophenol blue, 6% β-mercaptoethanol) were added and samples denatured for 10 min at 100°C. Protein content was assessed using a commercial kit (Bio-Rad, Hercules, CA, USA). Twenty micrograms of proteins were electrophoresed into a 10% sodium dodecyl sulfate (SDS)–polyacrylamide gel under reducing condition. The proteins were then transferred to polyvinylidene difluoride (PVDF) membrane (Amersham, Little Chalfont, UK). Proteins were visualized using as primary antibodies against PKCα, PKCβII and RACK1, and developed using enhanced chemiluminescence (Bio-Rad). The images of the blots were acquired with the Molecular Imager Gel Doc XR (Bio-Rad). Optical density (OD) of bands was calculated and analyzed by means of the Image Lab version 4.0.1 (Bio-Rad), normalized by β-tubulin, and results expressed as percentage of control.

4.4.5. Statistical analysis

All experiments were repeated at least three times. Data are expressed as mean ± SD. Statistical analysis was performed using InStat software version 7.0 (GraphPad Software, La Jolla, CA, USA). Statistical significance was determined by ANOVA followed by a multiple comparison test as indicated in the legends. Effects were designated significant at $p<0.05$.

References

[1] Z. Rozmer, P. Perjési, *Phytochem. Rev.* **2016**, *15*, 87-120.

[2] C. Zhuang, W. Zhang, C. Sheng, W. Zhang, C. Xing, Z. Miao, *Chem. Rev.* **2017**, *117*, 7762-7810.

[3] T. T. Komoto, T. M. Bernardes, T. B. Mesquita, L. F. B. Bortolotto, G. Silva, T. A. Bitencourt, S. J. Baek, M. Marins, A. L. Fachin, *Molecules* **2018**, *23*, 2018.

[4] H. Yin, J. Dong, Y. Cai, X. Shi, H. Wang, G. Liu, Y. Tang, J. Liu, L. Ma, *Eur. J. Med. Chem.* **2019**, *180*, 350-366.

[5] K. Silbermann, C. P. Shah, N. U. Sahu, K. Juvale, S. M. Stefan, P. S. Kharkar, M. Wiese, *Eur. J. Med. Chem.* **2019**, *164*, 193-213.

[6] D. Drutovic, M. Chripkova, M. Pilatova, P. Kruzliak, P. Perjesi, M. Sarissky, M. Lupi, G. Damia, M. Broggini, J. Mojzis, *Tumor Biol.* **2014**, *35*, 9967-9975.

[7] M. Mori, L. Tottone, D. Quaglio, N. Zhdanovskaya, C. Ingallina, M. Fusto, F. Ghirga, G. Peruzzi, M. E. Crestoni, F. Simeoni, F. Giulimondi, C. Talora, B. Botta, I. Screpanti, R. Palermo, *Scientific Reports* **2017**, *7*, 2213.
[8] D. Quaglio, N. Zhdanovskaya, G. Tobajas, V. Cuartas, S. Balducci, M. S. Christodoulou, G. Fabrizi, M. Gargantilla, E.-M. Priego, Á. Carmona Pestaña, D. Passarella, I. Screpanti, B. Botta, R. Palermo, M. Mori, F. Ghirga, M.-J. Pérez-Pérez, *ACS Med. Chem. Lett.* **2019**, *10*, 639-643.
[9] C.-H. Pui, L. L. Robison, A. T. Look, *The Lancet* **2008**, *371*, 1030-1043.
[10] E. Follini, M. Marchesini, G. Roti, *Int. J. Mol. Sci.* **2019**, *20*, 3021.
[11] J. Lei, Q. Li, Y. Gao, L. Zhao, Y. Liu, *Scientific Reports* **2016**, *6*, 33717.
[12] D. Ron, C. H. Chen, J. Caldwell, L. Jamieson, E. Orr, D. Mochly-Rosen, *PNAS* **1994**, *91*, 839-843.
[13] D. R. Adams, D. Ron, P. A. Kiely, *Cell Commun. Signaling* **2011**, *9*, 22.
[14] J. J. Li, D. Xie, *Oncogene* **2015**, *34*, 1890-1898.
[15] M. Nishikawa, S. Shirakawa, *Leuk. Lymphoma* **1992**, *8*, 201-211.
[16] M. R. Jirousek, P. G. Goekjian, *Expert Opin. Invest. Drugs* **2001**, *10*, 2117-2140.
[17] J. F. Beck, D. Brügger, K. Brischwein, C. Liu, P. Bader, D. Niethammer, V. Gekeler, *Jpn. J. Cancer Res.* **2001**, *92*, 896-903.
[18] M. Volm, F. Zintl, L. Edler, A. Sauerbrey, *Med. Pediatr. Oncol.* **1997**, *28*, 117-126.
[19] N. Isakov, *Semin. Cancer Biol.* **2018**, *48*, 36-52.
[20] D. Duff, A. Long, *Cell. Signalling* **2017**, *35*, 250-255.
[21] E. Buoso, M. Ronfani, M. Galasso, D. Ventura, E. Corsini, M. Racchi, *Pharmacol. Res.* **2019**, *143*, 17-26.
[22] M. S. Christodoulou, F. Nicoletti, K. Mangano, M. A. Chiacchio, G. Facchetti, I. Rimoldi, E. M. Beccalli, S. Giofrè, *Bioorg. Med. Chem. Lett.* **2020**, *30*, 126845.
[23] M. S. Christodoulou, M. Mori, R. Pantano, R. Alfonsi, P. Infante, M. Botta, G. Damia, F. Ricci, P. A. Sotiropoulou, S. Liekens, B. Botta, D. Passarella, *ChemPlusChem* **2015**, *80*, 938-943.
[24] I. Rimoldi, V. Coccè, G. Facchetti, G. Alessandri, A. T. Brini, F. Sisto, E. Parati, L. Cavicchini, G. Lucchini, F. Petrella, E. Ciusani, A. Pessina, *Biomed. Pharmacother.* **2018**, *108*, 111-118.
[25] M. S. Christodoulou, M. Zarate, F. Ricci, G. Damia, S. Pieraccini, F. Dapiaggi, M. Sironi, L. Lo Presti, A. N. García-Argáez, L. Dalla Via, D. Passarella, *Eur. J. Med. Chem.* **2016**, *118*, 79-89.
[26] M. S. Christodoulou, K. M. Kasiotis, N. Fokialakis, I. Tellitu, S. A. Haroutounian, *Tetrahedron Lett.* **2008**, *49*, 7100-7102.
[27] E. Gabriele, F. Porta, G. Facchetti, C. Galli, A. Gelain, F. Meneghetti, I. Rimoldi, S. Romeo, S. Villa, C. Ricci, N. Ferri, A. Asai, D. Barlocco, A. Sparatore, *Arkivoc* **2017**, *part ii*, 235-250.
[28] G. Facchetti, N. Ferri, M. G. Lupo, L. Giorgio, I. Rimoldi, *Eur. J. Inorg. Chem.* **2019**, *2019*, 3389-3395.
[29] M. S. Christodoulou, F. Zunino, V. Zuco, S. Borrelli, D. Comi, G. Fontana, M. Martinelli, J. B. Lorens, L. Evensen, M. Sironi, S. Pieraccini, L. Dalla Via, O. M. Gia, D. Passarella, *ChemMedChem* **2012**, *7*, 2134-2143.
[30] R. Gandolfi, G. Facchetti, M. S. Christodoulou, M. Fusè, F. Meneghetti, I. Rimoldi, *ChemistryOpen* **2018**, *7*, 393-400.
[31] A. Schiano Moriello, L. Luongo, F. Guida, M. S. Christodoulou, D. Perdicchia, S. Maione, D. Passarella, V. Di Marzo, L. De Petrocellis, *CNS & Neurological Disorders - Drug Targets- CNS & Neurological Disorders)* **2016**, *15*, 987-994.
[32] M. H. Kang, M. A. Smith, C. L. Morton, N. Keshelava, P. J. Houghton, C. P. Reynolds, *Pediatric Blood & Cancer* **2011**, *56*, 239-249.
[33] D. Z. Pozarowski P., *Analysis of Cell Cycle by Flow Cytometry*, Humana Press, **2004**

[34] D. Ron, D. Mochly-Rosen, *PNAS* **1995**, *92*, 492-496.
[35] OECD Guideline for Testing of Chemicals-Partition Coefficient (n-octanol/water), **High Performance Liquid Chromatography (HPLC) Method, 117, Adopted: 30.03.89**.
[36] F. Porta, G. Facchetti, N. Ferri, A. Gelain, F. Meneghetti, S. Villa, D. Barlocco, D. Masciocchi, A. Asai, N. Miyoshi, S. Marchianò, B.-M. Kwon, Y. Jin, V. Gandin, C. Marzano, I. Rimoldi, *Eur. J. Med. Chem.* **2017**, *131*, 196-206.
[37] I. Rimoldi, G. Facchetti, G. Lucchini, E. Castiglioni, S. Marchianò, N. Ferri, *Bioorg. Med. Chem.* **2017**, *25*, 1907-1913.
[38] L. C. Crowley, G. Chojnowski, N. J. Waterhouse, *Cold Spring Harbor Protocols* **2016**, *2016*, pdb.prot087247.