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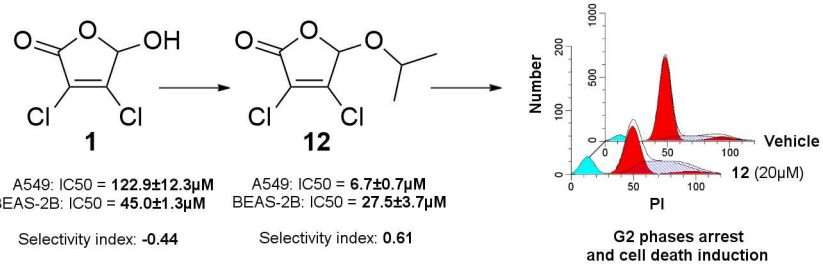

A549: IC50 = **122.9±12.3μM**
BEAS-2B: IC50 = **45.0±1.3μM**

Selectivity index: **-0.44**

A549: IC50 = **6.7±0.7μM**
BEAS-2B: IC50 = **27.5±3.7μM**

Selectivity index: **0.61**

G2 phases arrest and cell death induction

Simple 2(5*H*)-furanone derivatives with selective cytotoxicity towards non-small cell lung cancer cell line A549 – synthesis, structure-activity relationship and biological evaluation

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ABSTRACT

A series of 5-alkoxy derivatives of 3,4-dichloro-5-hydroxyfuran-2-(5*H*)-one (mucochloric acid, MCA) were obtained and subsequently subjected to modification in the C-4 position of 2(5*H*)-furanone ring. The cytotoxicity of newly synthesized compounds was evaluated in MTT assay against non-small cell lung cancer (A549) and healthy lung epithelial cell line (BEAS-2B). The derivatives containing a branched alkoxy substituent in the C-5 position demonstrated the highest anticancer properties, whereas modification of compounds in the C-4 position of 2(5*H*)-furanone ring only slightly improve their antiproliferative properties. Compounds **12** and **15** exhibited the best selectivity towards A549 cells and were also evaluated in a panel of cancer cell lines of different origin. Further investigation revealed that treatment of A549 cell line with compounds **12** and **15** led to G2 phase cell cycle arrest and

induction of caspase-independent cell death. Moreover, compound **12** was found to act synergistically with erlotinib.

Keywords: 2(5*H*)-Furanone, Nucleophilic substitution, Anticancer activity, Apoptosis

Abbreviations

DAPI (4',6-diamidino-2-phenylindole), dH_2O (deionized water), DMSO (dimethyl sulfoxide), ERB (erlotinib), ESI-MS (high-resolution electrospray ionization mass spectroscopy), FBS (fetal bovine serum), FITC (fluorescein isothiocyanate), IC_{50} (half maximal inhibitory concentration), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), NMR (nuclear magnetic resonance), NSCLC (non-small-cell lung carcinoma), PI (propidium iodide), SI (selectivity index), TLC (thin layer chromatography), TMS (tetramethylsilane).

1. Introduction

2(5*H*)-Furanone (2H-furan-5-on, γ-crotonolactone, **Figure 1**) is an organic compound that belongs to a group of α,β-unsaturated lactones, which are widely distributed in many natural products. [1-3] Compounds having 2(5*H*)-furanone skeleton in their structure are known to exhibit a wide range of biological activity, including antitumor, [3-8] antibacterial, [9-12] antifungal, [13,14] antiviral, [15] anti-inflammatory [16-19] and antioxidant. [17,20].

Several previous reports have confirmed the anticancer activity of compounds bearing a 2(5*H*)-furanone ring in their structure. [6,7] 5-Butoxy-3,4-dichloro-2(5*H*)-furanone (**Figure 1a**) is cytotoxic at high millimolar concentration in MAC 13 and MAC 16 murine colon cancer cell lines. [6] Further modifications of parent compound led to 3,4-dichloro-5-(oxirane-2-ylmethoxy)-2(5*H*)-furanone derivative (**Figure 1b**) with cytotoxicity in nanomolar range in the same cancer cell lines. [7] Peng and co-workers [21] showed that the compound (*E*)-3-(4-fluorophenyl)-*N*-(4-((2-methylene-5-oxo-2,5-dihydrofuran-3-yl)methyl)phenyl) acrylamide (**Figure 1c**) inhibits the formation of complex DNA-topoisomerase I. Also natural compounds known as rubrolides (**Figure 1d**) bearing 2(5*H*)-furanone moiety, possess anti-cancer activity in HT-29, MEL-28, P-388 and A549 cell lines. [22]

[Figure1]

These findings prompted us to investigate how simple modifications of 2(5*H*)-furanone scaffold affect their cytotoxic properties. Herein, we report the synthesis of mono- and disubstituted 2(5*H*)-furanone derivatives with the aim of establishment of structure-activity relationship (SAR) and exploration of their mechanism of action in non-small-cell lung cancer cell line.

2. Chemistry

The synthetic pathway leading to mono- and disubstituted 2(5*H*)-furanone derivatives is outlined in the **Scheme 1**. Our synthetic efforts started from mucochloric acid (MCA, **1**), which contains 2-(*5H*)-furanone and is readily available from commercial sources. Given the multiple functional groups in the different chemical environment, MCA offers accessibility to regioselective functionalization and thus rapid generation of structurally diverse compound libraries. First set of compounds, monosubstituted 5-alkoxy derivatives (**9**-**15**) were obtained in the reaction of MCA (**1**) with various alcohols (**2-8**) in toluene. The second library of compounds was obtained from **12**, **13** and **15** in the reaction with aromatic amines (**16-19**) or piperidine (**20**) in DMSO. This synthetic strategy afforded disubstituted compounds (**21**-**35**) in moderate to excellent yields ranging from 29 to 92%.

[Scheme 1]

The structure of all compounds was confirmed by ^{1}H NMR, ^{13}C NMR, high-resolution electrospray ionization mass spectroscopy (ESI-MS) and elemental analysis.

3. Results and Discussion

3.1. Cytotoxicity

Compound **1** and all obtained derivatives were evaluated for their *in vitro* cytotoxicity (**Table 1**) against non-small cell lung cancer (A549) and healthy lung epithelial cells (BEAS-2B). The IC_{50} values, defined as 50% cell growth inhibition in comparison to untreated control, were determined using MTT assay. [24] With exception of **21**, **24-26** and **35**, all compounds displayed significant improvement in the antiproliferative activity in the A549 cell line. Compounds having substituent only in the C-5 position of 2(*5H*)-furanone ring showed higher cytotoxicity than their counterparts bearing a modification on both C-4 and C-5 carbons, highlighting the importance of two halogens for the cytotoxic effect. The most active monosubstituted derivative **11** bearing propyl group, displayed IC_{50} value 3.6 µM and

was 34-times more potent than parent compound (IC_{50} value 122.9 μM). Among disubstituted derivatives, compound **33** appeared to be most active with IC_{50} value 13.3 μM, showing 9-fold improvement in anticancer activity, when compared the parent compound.

The major challenge in the development of novel anticancer compounds is their selectivity towards cancer cells. An optimal agent should affect only cancer cells and spare the healthy ones. Keeping this in mind, we assessed the cytotoxicity of all compounds also in healthy bronchial cell line BEAS-2B. The most potent compound from the series of monosubstituted derivatives **11** had almost the same IC_{50} values in both cancer and non-cancerous cell lines, indicating no selectivity towards cancer cells. Noteworthy, compounds **12**, **14** and **15**, having branched aliphatic chains, were more cytotoxic in A549 cell line with selectivity index (SI) values [25] 0.61, 0.22 and 0.88, respectively (**Table 1**). Based on these results, compounds **12** and **15** along with parent compound **1** were selected for further evaluation.

[Table 1]

Compounds **1**, **12** and **15** were also investigated in a panel of 12 human cancer cell lines of different origin (**Table 2**). In most of the tested cell lines, 5-substituted derivatives (**12** and **15**) show higher cytotoxicity than the parent compound. Only in HCT 116 wt cell line activities of novel derivatives were similar to the parent compound (8.3 μM vs. 7.2 μM). The most prominent improvement in the antiproliferative activity of both **12** and **15** was observed for hepatocellular carcinoma HepG2 cell line (6.5-fold) and in androgen-independent prostate cancer cell line DU 145 (5-fold).

[Table 2]

3.2. Cell cycle analysis

To test whether selected compounds (**1**, **12** and **15**) perturb the cell cycle progression of A549 cells, we evaluated their effects after continuous exposure in the two time regimes (24 and 72 h). All compounds were used at 10 μM concentration. Treatment of cells with **12** after 24 hours caused a slight increase in the percentage of cells in the sub-G1 (11.2%) and G2/M (23.4%) phase when compared to untreated control (**Figure 2A, 2C**). No significant changes in cell cycle were observed when the cells were treated with compounds **1** and **15**. To elucidate whether compounds trigger G2 phase arrest or cell cycle arrest in mitosis, we

counted mitotic nuclei. We found that all tested compounds did not induce the accumulation of A549 mitotic cells after 24 hours of treatment, indicating that cells are arrested in G2 phase (**Figure 2B**). To confirm cell cycle arrest in G2 phase at the molecular level, we analyzed the expression of cyclin B1 which governs transition of the cell cycle through mitosis. During G2 phase the level of cyclin B1 increases ensuring the progression of the cell cycle to mitosis [26-28]. We found that none of the compounds increased the expression level of cyclin B1 and compound **12** downregulated the cyclin B1 (**Figure 2D**). This clearly indicates that most active compounds arrest the cell cycle in early G2 phase rather than in mitosis.

[Figure 2]

3.3. Apoptosis induction

The increase of sub-G1 population in the cell cycle analysis after treatment with compound **12,** indicates DNA fragmentation and suggest that this compound trigger apoptosis in A549 cells. To investigate this first, we checked the percentage of the sub-G1 fraction after 72 h treatment of A549 cells with high doses of evaluated compounds (20 μM). As depicted in **Figure 3A, B**, **1** did not produce any significant changes in the cell cycle progression, nor in the sub-G1 content. Notably, a massive increase in the percentage of sub-G1 cells (~40%) was observed after treatment with compound **12**. Interestingly, a higher dose of compound **15** did not lead to any changes in the sub-G1 fraction. This strongly supports our hypothesis that compound **12** induced apoptosis in A549 cells.

To confirm that compound **12** trigger apoptosis in A549 cells, we employed an Annexin V/propidium iodide double staining assay using flow cytometry analysis (**Figure 3C, D**). [29] Continuous exposure of A549 cells with **12** at 20 μM concentration for 72 hours induced an almost 36-fold increase in the percentage of both early and late apoptotic cells (32.2%) compared to the untreated control (0.9%). Surprisingly, treatment of A549 cells with **15** at the same concentration resulted also in the increase of Annexin-positive cells (20.3%). As expected, compound **1** displayed no effects on apoptosis.

To elucidate the molecular mechanism of apoptosis triggered by compound **12** we determined the expression of 35 apoptosis-related proteins using commercially available Proteome Profiler Human Apoptosis Array Kit. On the basis of the densitometric analysis, we failed to observe any significant changes in the expression levels of both pro- and antiapoptotic proteins after 72 h treatment of A549 cells with **1**, **12** and **15** (**Figure 3E, F**). Additionally, none of the compounds triggered significant caspase-3 cleavage, which activity

is a hallmark of apoptotic cell death. Furthermore, we confirmed the expression of pro-caspase-3 using specific antibodies in western blot assay. Consistent with the previous results, the level of pro-caspase-3 did not change significantly for all tested compounds. Taking this into account we speculate, that A549 cells upon treatment of **12** underwent caspase-independent cell death which is more likely through observed cell cycle perturbations (G2 arrest) than regulation of apoptosis-related proteins.

[Figure 3]

3.4. Clonogenic potential

Next, we sought to test whether cells treated once with most active compounds retain their clonogenic potential [31]. Results indicate that, in contrast to compound **1**, both derivatives **12** and **15** were able to decrease the clonogenic potential of A549 cells, even at low micromolar concentration. The most prominent effect was observed for compound **12**. After 72 hours of treatment with **12,** only 40% and 15% of cells formed colonies at a concentration of 5 µM and 10 µM, respectively (**Figure 4**). This clearly indicates that compound **12** significantly reduced the clonogenic potential of A549 cells.

[Figure 4]

*3.5.Combination of **12** with erlotinib*

Erlotinib (Tarceva), an EGFR inhibitor, is a FDA-approved targeted drug for the treatment of NSCLC (non-small-cell lung carcinoma). However, previous reports have shown that A549 cell line, which harbors KRAS mutation, is highly resistant to erlotinib [32-34]. This is in agreement with our results that confirmed that A549 cells are insensitive to this agent. Taking into account that our best compound **12** decreased cell viability of A549 cells at low-micromolar concentrations, we reasoned to test the combination of compounds **12** with erlotinib.

First, we assessed the effect of the combination of different doses of erlotinib and compound **12** (10 µM) on the viability of A549 cells after 72 hours of treatment. We observed a slight synergistic effect but only when erlotinib was tested at relatively high concentrations (**Figure 5A**). Since increasing erlotinib dose did not result in any significant decrease in cell viability, for further evaluation we choose a combination of **12** and erlotinib at equimolar concentrations (10 µM). This combination decreased the number of colonies of A549 cells for

about 89% (relative to the vehicle). Compound tested alone at 10 μM decreased cell colony formation for 86% and 75% for compound **12** and erlotinib, respectively.

Further, we checked influence combination erlotinib and **12** on cell cycle (**Figure 5B**). After 72 hours treatment of A549 cells with combination 10 μM of **12** and 10 μM of erlotinib, we observed an increase of sub-G1 cells to 28.0% (**Figure 5C**). When tested alone, erlotinib caused only slight increase in the percentage of apoptotic cells (8%).

Finally, we assessed clonogenic potential after treatment of A549 cells with above compound combination (**Figure 5D**, **5E**). This led to significant reduction of clonogenic potential of cells when compared to single-agent treatment.

[Figure 5]

4. Conclusion

In summary, simple alkylation of mucochloric acid **1** to compounds **12** and **15**, resulted in novel potent derivatives with increased cytotoxic potency and better selectivity towards cancer cells in comparison to lead compound **1**. Observed cytotoxic effect resulted mainly from G2 phase cell cycle arrest in case of compound **12**, which was also able to trigger caspase-independent apoptosis in A549 cells. Moreover, after treatment A549 cells with compound **12**, a significant decrease in the clonogenic potential was also observed. The combination of **12** with erlotinib showed synergistic anticancer activity in comparison to treatment with each single agent in A549 cells. To summarise, our data indicate that simple compounds bearing 2(*5H*)-furanone scaffold might be valuable starting points for further development.

Although direct molecular targets of the most potent compound are not known yet, some clues about their potential mechanism of action may come from the recent paper of Wu and co-workers, who showed that compounds having biphenyl substituents on C-4 of 2(5*H*)-furanone moiety are able to bind to *c-myc* G4 DNA quadruplexes [8]. In this context, it will be interesting to evaluate our compounds for their ability to bind such DNA structures.

5. Experimental section

5.1. Chemistry

All reagents were obtained from commercially available sources (Merck, Acros Organics, Alpha Aesar, Sigma-Aldrich) and were used without purification. The synthetic pathways were shown in **Scheme 1**. NMR spectra were recorded on Agilent spectrometer 400

MHz and Varian spectrometer 600 MHz in DMSO-d_6 solution using tetramethylsilane (TMS) as an internal standard. Chemical shifts are reported as δ values (ppm). NMR solvent was purchased from ACROS Organics. Melting point measurements were performed in an open capillary using Stuart® SMP30 apparatus and are uncorrected. High-resolution electrospray ionization mass spectroscopy (ESI-MS) experiments were performed using a Waters Xevo G2 QTOF instrument equipped with an injection system (cone voltage 50 V; source 120°C). Elemental analysis experiments were performed using a Perkin Elmer 2400 Series II CHNS/O System. Analyses indicated by the symbols of the elements or functions were within ± 0.4 % of the theoretical values

5.1.1. 5-Substituted of 3,4-dichloro-2(5H)-furanone derivatives

3,4-Dichloro-5-hydroxy-2(5*H*)-furanone (**1**, 1eq.) was dissolved in anhydrous toluene (10:1, v toluene:mmol **1**) contained concentrated sulfuric acid (0.025 eq.). Next alcohol **2**-**8** (15 eq.) was added. The reaction mixture was refluxed until TLC indicated the total decay of the starting material (8-108 h). The volatiles were removed under diminished pressure. The residue was purified by silica gel column chromatography (toluene:ethyl acetate, 10:1 v/v) to give **9**-**15**.

*5.1.1.1. 3,4-Dichloro-5-methoxy-2(5H)-furanone (**9**)*

Colorless oil; yield: 81%; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.51 (s, 3H, -OC<u>H</u>$_3$), 6.23 (s, 1H, H-5); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 55.77 (C-1'), 101.44 (C-5), 123.20 (C-3), 147.44 (C-4), 162.93 (C-2); ESI-MS (CH_3OH): m/z 182.9616 $[M]^+$ (*m/z* calcd. 182.9616 $[M]^+$); Anal. Calcd. for $C_5H_4Cl_2O_3$: C, 32.82; H, 2.20. Found: C, 32.41; H, 1.95.

*5.1.1.2. 3,4-Dichloro-5-ethoxy-2(5H)-furanone (**10**)*

Colorless oil; yield 77%; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.21 (t, 3H, J = 7.2 Hz, -C<u>H</u>$_3$), 3.82 (q, 2H, J = 7.2 Hz, -OC<u>H</u>$_2$-), 6.23 (s, 1H, H-5); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 14.81 (C-2'), 65.27 (C-1'), 100.89 (C-5), 122.86 (C-3), 147.73 (C-4), 163.02 (C-2); ESI-MS (CH_3OH): *m/z* 218.9594 $[M+Na]^+$ (*m/z* calcd. 218.9592 $[M+Na]^+$) ; Anal. Calcd. for $C_6H_6Cl_2O_3$: C, 36.58; H, 3.07. Found: C, 36.25; H, 2.74.

*5.1.1.3. 3,4-Dichloro-5-propyloxy-2(5H)-furanone (**11**)*

Yellow oil; yield 80%; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 0.90 (t, 3H, J = 7.4 Hz, -C<u>H</u>$_3$), 1.57-1.63 (m, 2H, -C<u>H</u>$_2$-CH$_3$), 3.72 (t, 2H, J = 6.5 Hz, -OC<u>H</u>$_2$-), 6.28 (s, 1H, H-5); ^{13}C NMR

(100 MHz, DMSO-d_6) δ: 10.03 (C-3'), 22.15 (C-2'), 70.86 (C1'), 100.99 (C-5), 122.87 (C-3), 147.74 (C-4), 163.02 (C-2); ESI-MS (CH_3OH): *m/z* 210.9932 $[M]^+$ (*m/z* calcd. 210.9932 $[M]^+$); Anal. Calcd. for $C_7H_8Cl_2O_3$: C, 39.84; H, 3.82. Found: C, 40.21; H, 3.63.

*5.1.1.4. 3,4-Dichloro-5-isopropyloxy-2(5H)-furanone (**12**)*

White syrup; yield: 82%; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.22-1.24 (m, 6H, 2x-C<u>H</u>$_3$), 4.11-4.21 (m, 1H, -OC<u>H</u>-), 6.34 (s, H, H-5); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 22.16 (C-2'), 23.10 (C-2'), 75.13 (C-1'), 100.31 (C-5), 124.17 (C-3), 147.86 (C-4), 163.55 (C-2); ESI-MS (CH_3OH): *m/z* 210.9929 $[M]^+$ (*m/z* calcd. 210.9929 $[M]^+$); Anal. Calcd. for $C_7H_8Cl_2O_3$: C, 39.84; H, 3.82. Found: C, 39.92; H, 3.54.

*5.1.1.5. 5-n-Butyloxy-3,4-dichloro-2(5H)-furanone (**13**)*

Colorless oil; yield: 84%; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 0.89 (t, 3H, *J* = 7.2 Hz, -C<u>H</u>$_3$), 1.31-1.40 (m, 2H, -C<u>H</u>$_2$-CH$_3$), 1.53-1.60 (m, 2H, -OCH$_2$-C<u>H</u>$_2$), 3.77 (t, 2H, *J* = 6.4 Hz, -OC<u>H</u>$_2$-), 6.24 (s, 1H, H-5); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 13.44 (C-4'), 18.28 (C-3'), 30.75 (C-2'), 68.68 (C-1'), 103.22 (C-5), 117.51 (C-3), 144.56 (C-4), 164.17 (C-2); ESI-MS (CH_3OH): *m/z* 225.0085 $[M]^+$ (*m/z* calcd. 225.0085 $[M]^+$); Anal. Calcd. for $C_8H_{10}Cl_2O_3$: C, 42.69; H, 4.48. Found: C, 42.84; H, 4.67.

*5.1.1.6. 3,4-Dichloro-5-tert-butyloxy-2(5H)-furanone (**14**)*

White syrup; yield: 45%; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.31 (s, 9H, 3x-C<u>H</u>$_3$), 6.49 (s, 1H, H-5); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 27.70 (3 x C-2'), 78.68 (C-1'), 97.15 (C-5), 121.76 (C-3), 148.97 (C-4), 163.33 (C-2); ESI-MS (CH_3OH): *m/z* 246.9905 $[M+Na]^+$ (*m/z* calcd. 246.9905 $[M+Na]^+$); Anal. Calcd. for $C_8H_{10}Cl_2O_3$: C, 42.69; H, 4.48. Found: C, 43.09; H, 4.36.

*5.1.1.7. 3,4-Dichloro-5-neopentyloxy-2(5H)-furanone (**15**)*

Colorless oil; yield: 74%; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 0.91 (s, 9H, 3x-C<u>H</u>$_3$), 3.43 (s, 2H, -OC<u>H</u>$_2$-), 6.28 (s, 1H, H-5); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 25.96 (3 x C-3'), 31.47 (C-2'), 78.39 (C-1'), 101.16 (C-5), 122.94 (C-3), 147.70 (C-4), 162.97 (C-2); ESI-MS (CH_3OH): *m/z* 239.0242 $[M]^+$ (*m/z* calcd. 239.0242 $[M]^+$); Anal. Calcd. for $C_9H_{12}Cl_2O_3$: C, 45.21; H, 5.06. Found: C, 45.59; H, 5.22 .

5.1.2. 4- and 5-Substituted of 3,4-dichloro-2(5H)-furanone derivatives

Derivatives **12**, **13** and **15** (1eq.) were dissolved in anhydrous DMSO (5ml to 1eq.), next amine **16**-**20** (2eq.) was added. The reaction mixture was stirred at room temperature until TLC indicated the consumption of all the starting material (2-24h). The reaction mixture was quenched with cold water (10 ml) and extracted with dichloromethane (2x10 ml). The organic layer was dried (anhydrous $MgSO_4$). The solvent was removed under reduced pressure and the crude product was purified by column chromatography (toluene:ethyl acetate, 7:1 v/v) to give compounds **21**-**35**.

*5.1.2.1. 3-Chloro-4-phenylamino-5-isopropyloxy-2(5H)-furanone (**21**)*

Yellow solid; yield: 62%; m.p.=114-115^{o}C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 0.77 (d, 3H, J = 6.2 Hz, -C$\underline{H}_3$), 1.08 (d, 3H, J = 6.2 Hz, -C$\underline{H}_3$), 3.72-3.82 (m, 1H, -OC$\underline{H}$-), 6.36 (s, 1H, H-5), 7.20-7.25 (m, 3H, Ar-3'', Ar-4'', Ar-5''), 7.35-7.39 (m, 2H, Ar-2'', Ar-6''), 9.65 (s, 1H, -N$\underline{H}$); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 21.48 (C-2a'), 22.83 (C-2b'), 73.34 (C-1'), 87.84 (C-5), 97.04 (C-3), 123.43 (C-2'', C-6''), 125.12 (C-1''), 128.71 (C3'', C-5''), 137.87 (C-4''), 155.48 (C-4), 166.79 (C-2); ESI-MS (CH_3OH): *m/z* 268.0740 $[M]^+$ (*m/z* calcd. 268.0740 $[M]^+$); Anal. Calcd. for $C_{13}H_{14}ClNO_3$: C, 58.32; H, 5.27; N, 5.23. Found: C, 58.02; H, 5.03; N, 4.94.

*5.1.2.2. 4-(4'-Bromophenylamino)-3-chloro-5-isopropyloxy-2(5H)-furanone (**22**)*

Yellow solid; yield: 44%; m.p.=163-164^{o}C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 0.84 (d, 3H, J = 6.2 Hz, -C$\underline{H}_3$), 1.09 (d, 3H, J = 6.2 Hz, -C$\underline{H}_3$), 3.83 (sep, 1H, J = 6.2 Hz, -OC$\underline{H}$-), 6.36 (s, 1H, H-5), 7.18-7.21 (m, 2H, Ar-2'', Ar-6''), 7.52-7.56 (m, 2H, Ar-3'', Ar-5''), 9.69 (s, 1H, -N$\underline{H}$); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 21.55 (C-2a'), 22.83 (C-2b'), 73.34 (C-1'), 88.78 (C-5), 97.02 (C-3), 117.16 (C-4''), 125.22 (C-2'', C-6''), 131.51 (C-3'', C-5''), 137.32 (C-1''), 154.96 (C-4), 166.63 (C-2); ESI-MS (CH_3OH): *m/z* 345.9846 $[M]^+$ (*m/z* calcd. 345.9845 $[M]^+$); Anal. Calcd. for $C_{13}H_{13}BrClNO_3$: C, 45.05; H, 3.78; N, 4.04. Found: C, 44.87; H, 3.51; N, 3.67.

*5.1.2.3. 3-Chloro-4-(4'-chlorophenylamino)-5-isopropyloxy-2(5H)-furanone (**23**)*

Yellow solid; yield: 66%; m.p.=158-159^{o}C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 0.84 (d, 3H, J = 4.0 Hz, -C$\underline{H}_3$), 1.09 (d, 3H, J = 4.0 Hz, -C$\underline{H}_3$), 3.79-3.85 (m, 1H, -OC$\underline{H}$-), 6.37 (s, 1H, H-5), 7.25-7.27 (m, 2H, Ar-2'', Ar-6''), 7.41-7.43 (m, 2H, Ar-3'', Ar-5''), 9.71 (s, 1H, -NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 22.96 (C-2a', C-2b'), 73.42 (C-1'), 88.72 (C-3), 97.17 (C-5), 124.91 (C-2'', C-6''), 129.16 (C-3'', C-5''), 129.20 (C-4''), 136.96 (C-1''), 155.15 (C-4),

166.73 (C-2); ESI-MS (CH_3OH): *m/z* 302.0351 $[M]^+$ (*m/z* calcd. 302.0350 $[M]^+$); Anal. Calcd. for $C_{13}H_{13}Cl_2NO_3$: C, 52.68; H, 4.34; N, 4.64. Found: C, 51.95; H, 4.00; N, 4.54..

5.*1.2.4. 3-Chloro-4-(4'-fluorophenylamino)-5-isopropyloxy-2(5H)-furanone (**24**)*

Brown solid; yield: 56%; m.p.=128-130^{o}C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 0.81 (d, 3H, J = 6.2 Hz, -C<u>H</u>$_3$), 1.08 (d, 3H, J = 6.2 Hz, -C<u>H</u>$_3$), 3.78 (sep, 1H, J = 6.2 Hz, -OC<u>H</u>-), 6.30 (s, 1H, H-5), 7.18-7.24 (m, 2H, Ar-2'', Ar-6''), 7.26-7.31 (m, 2H, Ar-3'', Ar-5''), 9.62 (s, 1H, -N<u>H</u>); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 21.53 (C-2a'), 22.87 (C-2b'), 73.31 (C-1'), 87.67 (C-3), 96.90 (C-5), 115.46 (d, ^2J = 22.6 Hz, C-2'', C-6''), 125.91 (d, $^3J_{C\text{-}F}$ = 12.8 Hz, C-3'', C-5''), 134.23 (d, $^4J_{C\text{-}F}$ = 2.7 Hz, C-1''), 155.69 (C-4), 159.69 (d, $^1J_{C\text{-}F}$ = 240.9 Hz, C-4''), 166.80 (C-2); ESI-MS (CH_3OH): *m/z* 286.0646 $[M]^+$ (*m/z* calcd. 286.0646 $[M]^+$); Anal. Calcd. for $C_{13}H_{13}ClFNO_3$: C, 54.65; H, 4.59; N, 4.90. Found: C, 54.75; H, 4.39; N, 4.83.

*5.1.2.5. 3-Chloro-5-isopropyloxy-4-(piperidin-1-yl)-2(5H)-furanone (**25**)*

Brown solid; yield: 29%; m.p.=84-85^{o}C; ^{1}H NMR (600 MHz, DMSO-d_6) δ: 1.20 (t, 6H, J = 6.0 Hz, 2x-C<u>H</u>$_3$), 1.59-1.63 (m, 6H, -N(CH$_2$)$_2$(C<u>H</u>$_2$)$_3$), 3.58 (br.s., 4H, -N(C<u>H</u>$_2$)$_2$(CH$_2$)$_3$), 4.07 (sep, 1H, J = 6.0 Hz, -OC<u>H</u>-), 6.18 (s, 1H, H-5); ^{13}C NMR (150 MHz, DMSO-d_6) δ: 21.89 (C-4''), 23.20 (C-2a'), 23.30 (C-2b'), 25.82 (C-3'', C-5''), 48.34 (C-2'', C-6''), 72.52 (C-1'), 82.96 (C-3), 96.14 (C-5), 155.28 (C-4), 167.73 (C-2); ESI-MS (CH_3OH): *m/z* 260.1053 $[M]^+$ (*m/z* calcd. 260.1053 $[M]^+$); Anal. Calcd. for $C_{12}H_{18}ClNO_3$: C, 55.49; H, 6.99; N, 5.39. Found: C, 55.64; H, 7.15; N, 5.24.

*5.1.2.6. 5-n-Butyloxy-3-chloro-4-phenylamino-2(5H)-furanone (**26**)*

Pink solid; yield: 60%; m.p. = 54-56^{o}C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 0.74 (t, 3H, J = 7.4 Hz, -C<u>H</u>$_3$), 1.04-1.09 (m, 2H, -C<u>H</u>$_2$-CH$_3$), 1.29-1.33 (m, 2H, -OCH$_2$-C<u>H</u>$_2$-), 3.40-3.46 (m, 1H, H$_a$, -OC<u>H</u>$_2$-), 3.57-3.32 (m, 1H, H$_b$, -OC<u>H</u>$_2$-), 6.32 (s, 1H, H-5), 7.17-7.24 (m, 3H, Ar-2'', Ar-4'', Ar-6''), 7.34-7.38 (m, 2H, Ar-3'', Ar-5''), 9.73 (s, 1H, -N<u>H</u>); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 13.43 (C-4'), 18.16 (C-3'), 30.66 (C-2'), 68.13 (C-1'), 87.94 (C-3), 97.52 (C-5), 123.03 (C-2', C-6'''), 125.00 (C-4''), 128.69 (C-3'', C-5''), 137.75 (C-1''), 154.76 (C-4), 166.70 (C-2); ESI-MS (CH_3OH): *m/z* 282.0897 $[M]^+$ (*m/z* calcd. 282.0897 $[M]^+$); Anal. Calcd. for $C_{14}H_{16}ClNO_3$: C, 59.68; H, 5.72; N, 497. Found: C, 59.78; H, 5.52; N, 5.01.

*5.1.2.7. 4-(4'-Bromophenylamino)-5-n-butyloxy-3-chloro-2(5H)-furanone (**27**)*

Yellow solid; yield: 55%; m.p.=114-115°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 0.75 (t, 3H, J = 7.4 Hz, -C<u>H</u>$_3$), 1.02-1.10 (m, 2H, -C<u>H</u>$_2$-CH$_3$), 1.29-1.36 (m, 2H, -OCH$_2$-C<u>H</u>$_2$-), 3.43-3.49 (m, 1H, H$_a$, -OC<u>H</u>$_2$-), 3.59-3.64 (m, 1H, H$_b$, -OC<u>H</u>$_2$-), 6.33 (s, 1H, H-5), 7.17-7.22 (m, 2H, Ar-2'', Ar-6''), 7.55-7.56 (m, 2H, Ar-3'', Ar-5''), 9.78 (s, 1H, -<u>N</u>H); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 13.46 (C-4'), 18.20 (C-3'), 30.70 (C-2'), 68.31 (C-1'), 88.95 (C-3), 97.56 (C-5), 117.21 (C-4''), 124.92 (C-2'',C-6''), 131.61 (C-3'', C-5''), 137.35 (C-1''), 154.51 (C-4), 166.59 (C-2); ESI-MS (CH_3OH): *m/z* 360.0002 $[M]^+$ (*m/z* calcd. 360.0002 $[M]^+$); Anal. Calcd. for $C_{14}H_{15}BrClNO_3$: C, 46.63; H, 4.19; N, 3.88. Found: C, 46.84; H, 4.00; N, 3.72.

*5.1.2.8. 5-n-Butyloxy-3-chloro-4-(4'-chlorophenylamino)-2(5H)-furanone (**28**)*

Yellow solid; yield: 20%; m.p.=107-109°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 0.75 (t, 3H, J = 7.4 Hz, -C<u>H</u>$_3$), 1.02-1.11 (m, 2H, -C<u>H</u>$_2$-CH$_3$), 1.29-1.36 (m, 2H, -OCH$_2$-C<u>H</u>$_2$-), 3.43-3.49 (m, 1H, H$_a$, -OC<u>H</u>$_2$-), 3.59-3.64 (m, 1H, H$_b$, -OC<u>H</u>$_2$-), 6.33 (s, 1H, H-5), 7.24-7.27 (m, 2H, Ar-2'', Ar-6''), 7.40-7.43 (m, 2H, Ar-3'', Ar-5''), 9.79 (s, 1H, -N<u>H</u>); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 13.48 (C-4'), 18.21 (C-3'), 30.72 (C-2'), 68.26 (C-1'), 88.82 (C-3), 97.56 (C-5), 124.65 (C-2'', C-6''), 128.71 (C-3'', C-5''), 129.12 (C-4''), 136.92 (C-1''), 154.62 (C-4), 166.63 (C-2); ESI-MS (CH_3OH): *m/z* 316.0507 $[M]^+$ (*m/z* calcd. 316.0507 $[M]^+$); Anal. Calcd. for $C_{14}H_{15}C_{l2}FNO_3$: C, 53.18; H, 4.78; N, 4.43. Found: C, 53.58; H, 4.79; N, 4.36.

*5.1.2.9. 5-n-Butyloxy-3-chloro-4-(4'-fluorophenylamino)-2(5H)-furanone (**29**)*

Yellow solid; yield: 60%; m.p.=77-78°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 0.75 (t, 3H, J = 7.4 Hz, -C<u>H</u>$_3$), 1.06-1.11 (m, 2H, -C<u>H</u>$_2$-CH$_3$), 1.29-1.36 (m, 2H, -OCH$_2$-C<u>H</u>$_2$-), 3.40-3.45 (m, 1H, H$_a$, -OC<u>H</u>$_2$-), 3.57-3.62 (m, 1H, H$_b$, -OC<u>H</u>$_2$-), 6.27 (s, 1H, H-5), 7.18-7.22 (m, 2H, Ar-2'', Ar-6''), 7.26-7.30 (m, 2H, Ar-3'', Ar-5''), 9.71 (s, 1H, -N<u>H</u>); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 13.46 (C-4'), 18.23 (C-3'), 30.72 (C-2'), 68.17 (C-1'), 87.77 (C-3), 97.45 (C-5), 115.48 (d, $^2J_{C\text{-}F}$ = 18 Hz, C-2'', C-6''), 125.49 (d, $^3J_{C\text{-}F}$ = 8.4 Hz, C-3'', C-5''), 134.15 (d, $^4J_{C\text{-}F}$ = 2.7 Hz, C-1''), 155.05 (C-4), 159.70 (d, $^1J_{C\text{-}F}$ = 238.2 Hz, C-4''), 166.73 (C-2); ESI-MS (CH_3OH): *m/z* 286.0646 $[M]^+$ (*m/z* calcd. 286.0646 $[M]^+$); Anal. Calcd. for $C_{14}H_{15}ClFO_3$: C, 56.10; H, 5.04; N, 4.67. Found: C, 55.75; H, 4.88; N, 4.57.

*5.1.2.10. 5-n-Butyloxy-3-chloro-4-(piperidin-1-yl)-2(5H)-furanone (**30**)*

Yellow oil; yield: 64%; oil; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 0.88 (t, 3H, J = 7.4 Hz, -C<u>H</u>$_3$), 1.31-1.38 (m, 2H, -C<u>H</u>$_2$-CH$_3$), 1.51-1.62 (m, 8H, -OCH$_2$-C<u>H</u>$_2$-, -N(CH$_2$)$_2$(C<u>H</u>$_2$)$_3$), 3.60-3.71 (m, 6H, -OC<u>H</u>$_2$-, -N(C<u>H</u>$_2$)$_2$(CH$_2$)$_3$), 6.11 (s, 1H, H-5); ^{13}C NMR (100 MHz, DMSO-d_6) δ:

13.50 (C-4'), 18.58 (C-3'), 23.23 (C-4''), 25.83 (C-3'', C-5''), 30.90 (C-2'), 48.38 (C-2'', C-6''), 67.51 (C-1'), 82.97 (C-3), 96.72 (C-5), 154.84 (C-4), 167.60 (C-2); ESI-MS (CH_3OH): *m/z* 274.1210 $[M]^+$ (*m/z* calcd. 274.1210 $[M]^+$); Anal. Calcd. for $C_{13}H_{20}ClNO_3$: C, 57.04; H, 7.36; N, 5.12. Found: C, 57.44; H, 7.63; N, 5.11.

*5.1.2.11. 3-Chloro-4-phenylamino-5-neopentyloxy-2(5H)-furanone (**31**)*

Yellow solid; yield: 76%; m.p.=166-168°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 0.63 (s, 9H, 3x-C$\underline{H}_3$), 3.03 (d, 1H, *J* = 8.4 Hz, H_a, -OC$\underline{H}_2$-), 3.25 (d, 1H, *J* = 8.4Hz, H_b, -OC$\underline{H}_2$-), 6.42 (s, 1H, H-5), 7.14-7.18 (m, 1H, Ar-4''), 7.24-7.25 (m, 2H, Ar-2'', Ar-6''), 7.33-7.37 (m, 2H, Ar-3'', Ar-5''), 9.71 (s, 1H, -N$\underline{H}$); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 25.89 (3xC-3'), 30.90 (C-2'), 77.48 (C-1'), 88.20 (C-3), 97.24 (C-5), 122.79 (C-4''), 124.94 (C-2', C-6'''), 128.82 (C-3'', C-5''), 138.04 (C-1''), 155.08 (C-4), 166.55 (C-2); ESI-MS (CH_3OH): *m/z* 296.1053 $[M]^+$ (*m/z calcd.* 296.1053 $[M]^+$); Anal. Calcd. for $C_{15}H_{18}ClNO_3$: C, 60.91; H, 6.13; N, 4.74. Found: C, 60.73; H, 6.32; N, 4.61.

*5.1.2.12. 4-(4'-Bromophenylamino)-3-chloro-5-neopentyloxy-2(5H)-furanone (**32**)*

Yellow solid; yield: 50%; m.p.=143-145°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 0.65 (s, 9H, 3x-C$\underline{H}_3$), 3.07 (d, 1H, *J* = 8.4 Hz, H_a, -OC$\underline{H}_2$-) 3.25 (d, 1H, *J* = 8.4Hz, H_b, -OC$\underline{H}_2$-), 6.42 (s, 1H, H-5), 7.20-7.24 (m, 2H, Ar-2'', Ar-6''), 7.51-7.55 (m, 2H, Ar-3'', Ar-5''), 9.77 (s, 1H, -N$\underline{H}$); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 25.83 (3xC-3'), 30.92 (C-2'), 77.49 (C-1'), 89.10 (C-3), 97.21 (C-5), 117.04 (C-4''), 124.61 (C-2'', C-6''), 131.64 (C-3'', C-5''), 137.53 (C-1''), 154.70 (C-4), 166.38 (C-2); ESI-MS (CH_3OH): *m/z* 374.0159 $[M]^+$ (*m/z* calcd. 374.0158 $[M]^+$); Anal. Calcd. for $C_{15}H_{17}BrClNO_3$: C, 48.09; H, 4.57; N, 3.74. Found: C, 48.36; H, 4.49; N, 3.65.

*5.1.2.13. 5-Chloro-4-(4'-chlorophenylamino)-5-neopentyloxy-2(5H)-furanone (**33**)*

White solid; yield: 60%; m.p.=111-113°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 0.65 (s, 9H, 3x-C$\underline{H}_3$), 3.07 (d, 1H, *J* = 8.4 Hz, H_a, -OC$\underline{H}_2$-), 3.25 (d, 1H, *J* = 8.4 Hz, H_b, -OC$\underline{H}_2$-), 6.42 (s, 1H, H-5), 7.27-7.30 (m, 2H, Ar-2'', Ar-6''), 7.38-7.42 (m, 2H, Ar-3'', Ar-5''), 9.78 (s, 1H, -N$\underline{H}$); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 25.90 (3xC-3'), 30.99 (C-2'), 77.55 (C-1'), 89.05 (C-3), 97.27 (C-5), 124.38 (C-2'', C-6''), 128.79 (C-3'', C-5''), 129.04 (C-4''), 137.17 (C-1''), 154.86 (C-4), 166.47 (C-2); ESI-MS (CH_3OH): *m/z* 330.0663 $[M]^+$ (*m/z* calcd. 330.0663 $[M]^+$); Anal. Calcd. for $C_{15}H_{17}Cl_2NO_3$: C, 54.56; H, 5.19; N, 4.24. Found: C, 54.87; H, 5.22; N, 4.06.

*5.1.2.14. 3-Chloro-4-(4'-fluorophenylamino)-5-neopentyloxy-2(5H)-furanone (**34**)*

White solid; yield: 60%; m.p.=113-115°C; ^{1}H NMR (600 MHz, DMSO-d_6) δ: 0.65 (s, 9H, 3x-C<u>H</u>$_3$), 3.03 (d, 1H, J = 8.4 Hz, H_a, -OC<u>H</u>$_2$-), 3.25 (d, 1H, J = 8.4 Hz, H_b, -OC<u>H</u>$_2$-), 6.38 (s, 1H, H-5), 7.17-7.21 (m, 2H, Ar-2'', Ar-6''), 7.29-7.32 (m, 2H, Ar-3'', Ar-5''), 9.72 (s, 1H, -N<u>H</u>); ^{13}C NMR (150 MHz, DMSO-d_6) δ: 25.88 (3xC-3'), 30.94 (C-2'), 77.46 (C-1'), 88.00 (C-3), 97.13 (C-5), 115.54 (d, $^{2}J_{C\text{-}F}$ = 22.6 Hz, C-2'', C-6''), 125.13 (d, $^{3}J_{C\text{-}F}$ = 8.4 Hz, C-3'', C-5''), 134.39 (C-1''), 155.27 (C-4), 159.56 (d, $^{1}J_{C\text{-}F}$ = 240.9 Hz, C-4''), 166.54 (C-2); ESI-MS (CH_3OH): *m/z* 314.0959 $[M]^+$ (*m/z* calcd. 314.0959 $[M]^+$); Anal. Calcd. for $C_{15}H_{17}ClFNO_3$: C, 57.42; H, 5.46; N, 4.46. Found: C, 57.66; H, 5.50; N, 4.40.

*5.1.2.15. 3-Chloro-5-neopentyloxy-4-(piperidin-1-yl)-2(5H)-furanone (**35**)*

White solid; yield: 92%; m.p.=95-97°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 0.89 (s, 9H, 3xC<u>H</u>$_3$), 1.60-1.62 (m, 6H, -N(CH$_2$)$_2$(C<u>H</u>$_2$)$_3$), 3.23 (d, 2H, J = 5.2 Hz, -OC<u>H</u>$_2$-), 3.61 (br.s., 4H, -N(C<u>H</u>$_2$)$_2$(CH$_2$)$_3$), 6.11 (s, 1H, H-5); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 23.20 (3xC-3'), 25.82 (C-3'', C-5''), 26.22 (C-2'', C-6''), 31.16 (C-2'), 48.30 (C-4''), 77.20 (C-1'), 83.10 (C-3), 96.60 (C-5), 154.65 (C-4), 167.46 (C-2); ESI-MS (CH_3OH): *m/z* 288.1368 $[M]^+$ (*m/z* calcd. 288.1366 $[M]^+$); Anal. Calcd. for $C_{14}H_{22}ClNO_3$: C, 58.43; H, 7.71; N, 4.87. Found: C, 58.40; H, 7.78; N, 4.81.

5.2. Cell lines

Human cells lines: non-small lung carcinoma (A549), bronchial epithelial cells (BEAS-2B), human colon cancer (HCT-116 wt, HT-29, LoVo), human hepatoma cancer (Hep3B, HepG2), prostate cancer (PC-3, DU 145) breast cancer (MCF-7) and osteosarcoma (U2OS, SJSA-1) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). HCT 116 -/- p53 (deleted both p53 alleles) were a gift from Dr. Bert Vogelstein. Cells were grown in DMEM with high glucose medium (Sigma-Aldrich, Germany) supplemented with 10% (v/v) inactivated fetal bovine serum (FBS) (EURx, Poland) and 1% antibiotics (10000 μg/ml of streptomycin and 10000 units/ml of penicillin) (Sigma-Aldrich, Germany) at 37°C in humidified atmosphere with 5% CO_2. In order to achieve the desired concentrations of the tested compounds, the stock solutions were mixed with fresh medium. Final DMSO concentration in culture media was less than 0.4%. Control cells in all experiments were grown in medium containing 0.4% DMSO. This concentration of DMSO did not alter cell growth rate and cell-cycle, as compared with the vehicle-free medium.

5.3. MTT assay

Cytotoxicity of compounds was assessed with the MTT (3-[4,5-dimethylthiazol-2-y]2,5-diphenyltetrazolium bromide) (Sigma-Aldrich, Germany) test. Cells were seeded on the 96-well plates (Nunc, Germany) with $2x10^3$ cells per well. After 24 hours of incubation, the tested agents were added and incubated for 72 hours. After that the MTT solution (0.5mg/ml) was added. After 3 hours of incubation, the MTT solution was removed and the formazan crystals were dissolved in 2-propanol (Avantor, Poland) containing 0.04 M hydrochloric acid (Avantor, Poland). Finally, the absorbance was measured at the 570 nm wavelength using multiwall plate reader (BioTek, USA). The experiment was done in at least three independent replicates. Cell viability was expressed as a percentage versus vehicle control. IC_{50} was defined as a concentration of a drug that decreased cell viability by 50% relative to the untreated control.

5.4. Cell cycle analysis

Cells ($5x10^4$ per well) were seeded on 6-well plate (Nunc, Germany) and allowed to attach for 24 h. Then the medium was replaced with tested compounds and the cells were incubated for 24 or 72 h. Floating cells were collected and added to adherent cells, harvested by trypsinization. Cells were washed with PBS (phosphate buffered saline) (Sigma-Aldrich, Germany) and then fixed in ice-cold ethanol (70%) for 30 min, treated with RNase A (100 μg/mL, EURx, Poland) and stained with propidium iodide (PI) (100 μg/mL, Acros Organics, Belgium). The DNA content was analyzed using a Becton Dickinson FACS Aria III sorter (BD Company, USA). Experiments were repeated at least twice. Data were analyzed using ModFit LT^{TM} Software (Verity Software House, USA).

5.5. Annexin V-FITC apoptosis assay

$5x10^4$ cells per well were seeded on 6-well plate (Nunc, Germany) and grown for 24 h. Then the medium was replaced with tested compounds and cells were incubated for 24 or 72 h. Floating cells were collected and added to adherent cells, harvested by trypsinization. Afterwards, we followed instructions from the manufacturer of the Annexin V-FITC Apoptosis Detection Kit (Biotool, USA). Fluorescence was measured using a Becton Dickinson FACS Aria III sorter (BD Company, USA). Experiments were repeated at least twice. Data were analyzed using Flowing Software (Cell Imaging Core, Turku Centre for Biotechnology, Turku).

5.6. Analysis of the expression of apoptosis-related proteins

The levels of expression of apoptosis-related proteins were evaluated using Human Apoptosis Array Kit (R&D Systems, United Kingdom). $2.5x10^5$ cells were seeded on 100mm dishes (Nunc, Germany) and allowed to attach for 24 h. Then the medium was replaced with tested compounds and the cells were incubated for 72 h. Next cells were lysed and the lysates were clarified by centrifugation for 30 min at 14,000×g. The total protein concentration was determined using Protein Quantification Kit (Sigma-Aldrich, Germany). Equivalent amounts of protein were diluted and incubated with the array membranes. The membranes were then scanned in the G:Box transilluminator (Syngene, United Kingdom) and the protein expression levels were quantified by densitometric analysis.

5.7. Western blot analysis

$2.5x10^5$ cells were seeded on 100mm dishes (Nunc, Germany) and allowed to attach for 24 h. Then the medium was replaced with tested compounds and the cells were incubated for 72 h. Cells were washed with cold PBS (Sigma-Aldrich, Germany) and them 300µl lysis buffer (50mM Tris pH 7.5, 150mM NaCl, 1% NP40, 50mM PMSF, 1mM DTT, 1x Inhibitor Protease Cocktail) was added to each dish. Cells lysates were rocked for 20 min at 4^oC and centrifuged at 15000xg for 30 min. The concentration of proteins in the supernatants were determined using Protein Quantification Kit (Sigma-Aldrich, Germany). 60µg of the protein was loaded into each lane. SDS-polyacrylamide gel electrophoresis was carried out in 12% gel. After electrophoresis, proteins were transferred to nitrocellulose membranes and blocked with 5% skim milk in TBST buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Tween 20) for 45 minutes at 4^oC. The primary antibodies solutions (Cell Signaling Technology, United States) were added to the membranes and were gently rocked overnight at 4 °C (1:1000 dilutions). Next day, membranes were incubated with appropriate HRP-conjugated secondary antibodies (Cell Signaling Technology, USA) and then ECL substrate was added. The membranes were then scanned in the G:Box transilluminator (Syngene, United Kingdom) and the protein expression levels were quantified by densitometric analysis.

5.8. Mitotic index

Cells ($5x10^4$ cells per well) were grown on 6-well plate (Nunc, USA) for 24 h. Then the medium was replaced with the one containing tested agents. Cells were incubated for 24 hours in the cell culture incubator. After washing with cold, PBS cells were fixed in ice-cold

methanol (−20°C) for 10 minutes, air-dried, washed with PBS, and stained with 2 μg/ml 4′-6′-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich, Germany). Slides were mounted in Fluoromount™ Aqueous Mounting Medium (Sigma-Aldrich, Germany). 1000 DAPI-stained cells were examined in each well by fluorescence microscopy (Zeiss, USA) using the ×40 objective.

5.9. Clonogenic assay

Cells ($5x10^4$ cells per well) were plated in 6-well plate (Nunc, Germany) and treated with the tested compounds for 24 and 72 h. Adherent cells were harvested by trypsinization. Cells were re-seeded then on the new 6-well plate (Nunc, Germany) with a density of $2x10^3$ cells per well. After 10 days cells were washed with PBS (Sigma-Aldrich, Germany), next cells were fixed in ice-cold ethanol (-20^{o}C) for 3 minutes, air-dried, washed again with PBS and stained with 0.01% crystal violet (Sigma-Aldrich, Germany) in dH_2O (deionized water) for 15 minutes. Next plates were washed dH_2O and allowed to dry. Colonies were counted with a microscope (Zeiss, USA). Aggregates of 30 cells or more were considered colonies.

6. Statistical analysis

The results were expressed as means ± S.D. and performed by T-student test, p values≤0.05 being considered statistically significant.

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Figure Captions

Figure 1. Structure of compounds bearing 2(5*H*)-furanone with anticancer activity (**a**) 5-butoxy-3,4-dichloro-2(5*H*)-furanone, (**b**) 3,4-dichloro-5-(oxiran-2-ylmethoxy)-2(5*H*)-furanone, (**c**) (*E*)-3-(4-fluorophenyl)-*N*-(4-((2-methylene-5-oxo-2,5-dihydrofuran-3-yl)methyl)phenyl)acrylamide and (**d**) rubrolide M. [6, 7, 19, 20]

Figure 2. Compound **12** perturbs cell cycle in A549 cells. (**A**) Cells cycle after 24 and 72 h treatment with **1**, **12** and **15** against (**B**) Mitotic index after 24 hours treatment with **1**, **12** and **15**. (**C**) Percentage of cells in sub-G1 after 24 and 72 h treatment with **1**, **12** and **15** (**D**) Western blot analysis of cyclin B1 expression after 72 h treatment with 10 µM of **1**, **12** and **15**. β-actin was used as a loading control.

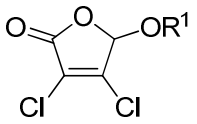
Figure 3. Compound **12** promotes cell death in A549 cells. (**A**) Cells cycle after 72 h treatment with 20 µM of **1**, **12** and **15**, (**B**) Comparison of the percentage of cells in sub-G1 phase, (**C**) Induction of apoptosis after 72 h treatment with 20 µM of **1**, **12** and **15** detected in annexin V-FITC/PI assay (**D**) Comparison of the percentage of cells in early and late apoptosis. (**E**) Changes of apoptosis-related proteins found in A549 cell line after 72 hours treatment with 20 µM of **1**, **12** and **15**. (**F**) Pixel density of apoptosis-proteins after 72 hours treatment **1**, **12** and **15** against A549 cell lines. (**G**) Western blot analysis of procaspase-3 expression in A549 cells treated with 20 µM of **1**, **12** and **15** after 72 hours. β-actin was used as a loading control.

Figure 4. Compound **12** decreases the clonogenic potential of A549 cells. (**A**) Representative colony formation images of the non-small cell lung cancer after treatment with compounds (**B**) The fraction of surviving cells after 72 hours treatment with 10 µM **1**, **12** and **15** against A549 cell line. (**C**) Number of colonies formed after 72 hours treatment with 10 µM **1, 12** and **15** against A549 cell line.

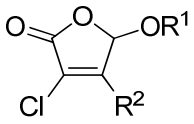
Figure 5. Compound **12** act synergistically with erlotinib in A549 cells. (**A**) Cell viability after 72 hours treatment of A549 cells with **12**, **erlotinib** (**ERB**) and their combination. (**B**) Cell cycle and (**C**) percentage of cells in sub-G1 after 72 hours treatment with **12**, **ERB** and their combination at 10 µM concentration. (**D**) Representative colony formation images of A549 cells after treatment with indicated compounds. (**E**) Number of colonies formed after 72 hours treatment with 10 µM **1, 12**, **ERB** and their combination.

Scheme 1. Synthesis of 5- and 4,5- substituted mucochloric acid (**1**) derivatives; a) toluene, conc. H_2SO_4, reflux b) DMSO, room temperature. Numbers in brackets indicate starting alcohol (**2-8**) or amine (**16-20**). [6, 7, 12, 23]

Table 1. Cytotoxicity and selectivity index (SI) of 3,4-dichloro-5-hydroxy-2(5*H*)-furanone derivatives against A549 and BEAS-2B cell lines



1, 9-15



21-35

Compounds	R^1	R^2	IC_{50}[a] for cell lines (μM)		SI[b]
			A549	BEAS-2B	
1	H	-	122.9 ± 12.3	45.0 ± 1.3	-0.44
9	Me	-	5.1 ± 0.1	4.6 ± 0.5	0.05
10	Et	-	6.3 ± 0.1	3.8 ± 0.4	0.22
11	Pr	-	3.6 ± 0.2	3.5± 0.1	-0.02
12	*i*-Pr	-	6.7 ± 0.7	27.5 ± 3.7	0.61
13	*n*-Bu	-	22.5 ± 4.5	22.3 ± 4.0	0.00
14	*tert*-Bu	-	14.0 ± 2.0	23.1 ± 4.9	0.22
15	neopentyl	-	7.7 ± 1.6	59.0 ± 9.1	0.88
21	*i*-Pr	C_6H_5-NH-	>400[c]	>400[c]	nc[d]
22	*i*-Pr	4-Br-C_6H_4-NH-	95.9 ± 3.8	201.9 ± 8.4	0.32
23	*i*-Pr	4-Cl-C_6H_4-NH-	39.6 ± 6.0	77.0 ± 9.4	0.29
24	*i*-Pr	4-F-C_6H_4-NH-	185.5 ± 8.9	>400[c]	nc[d]
25	*i*-Pr	piperidin-1-yl	>400[c]	>400[c]	nc[d]
26	*n*-Bu	C_6H_5-NH-	175.4 ± 18.7	206.1 ± 3.2	0.07
27	*n*-Bu	4-Br-C_6H_4-NH-	46.5 ± 6.7	42.7 ± 7.0	-0.04
28	*n*-Bu	4-Cl-C_6H_4-NH-	58.1 ± 1.1	60.8 ± 11.2	0.02
29	*n*-Bu	4-F-C_6H_4-NH-	78.5 ± 5.2	122.4 ± 3.2	0.19
30	*n*-Bu	piperidin-1-yl	93.3 ± 6.3	172.1 ± 1.8	0.27
31	neopentyl	C_6H_5-NH-	76.2 ± 0.4	76.2 ± 1.6	0.00
32	neopentyl	4-Br-C_6H_4-NH-	28.0 ± 1.5	30.9 ± 0.2	0.23
33	neopentyl	4-Cl-C_6H_4-NH-	13.3 ± 2.0	30.3 ± 4.5	0.36
34	neopentyl	4-F-C_6H_4-NH-	26.7 ± 3.5	47.6 ± 1.1	0.25
35	neopentyl	piperidin-1-yl	>400[c]	>400[c]	nc[d]

[a] IC_{50} is the concentration of a drug that decreased cell viability by 50% relative to the untreated control.

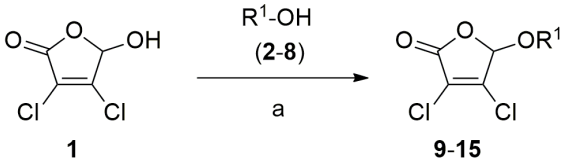
[b] SI has been calculated as a logarithm of a ratio of IC_{50} value for healthy cells (BEAS-2B) and the IC_{50} value for cancer cells (A549). [25]

[c] The maximum use of the compound concentration.

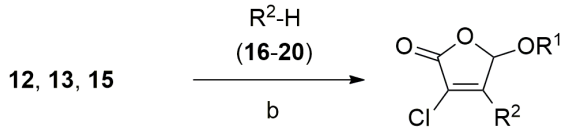
[d] nc (not calculated) – the SI could not be calculated due to the lack of the IC_{50} value for healthy/cancer cells.

Table 2. Cytotoxicity of compounds **1**, **12** and **15** against panel of cancer cell lines

Cell lines	IC_{50} for compounds (μM)		
	1	**12**	**15**
HCT 116 wt	8.3 ± 1.1	7.2 ± 1.6	7.2 ± 0.1
HCT 116 p53-/-	10.8 ± 1.0	6.8 ± 0.3	6.2 ± 0.6
HT-29	21.8 ± 0.8	7.2 ± 0.1	7.2 ± 0.1
LoVo	17.2 ± 2.3	2.8 ± 0.1	7.3 ± 0.6
Hep3B	85.1 ± 13.5	19.8 ± 1.5	27.8 ± 3.2
HepG2	111.3 ± 18.1	16.8 ± 1.0	17.6 ± 0.6
U2OS	58.5 ± 3.1	21.1 ± 0.1	38.1 ± 3.9
SJSA-1	39.4 ± 1.8	7.7 ± 0.1	11.1 ± 1.9
MCF-7	32.3 ± 4.4	12.4 ± 2.0	11.2 ± 1.5
PC-3	24.7 ± 1.1	4.8 ± 0.3	6.0 ± 0.3
DU 145	129.7 ± 8.0	25.4 ± 2.8	26.8 ± 1.7

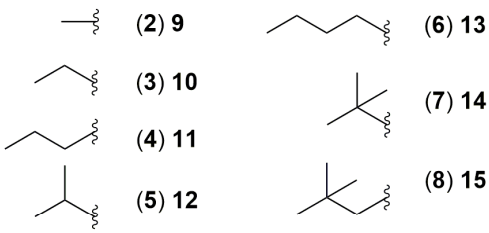


1 **9-15**



21-35

R^1:



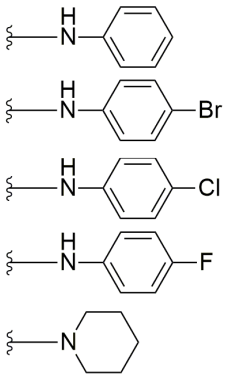
(**2**) **9** (**6**) **13**

(**3**) **10** (**7**) **14**

(**4**) **11** (**8**) **15**

(**5**) **12**

R^2:



(**16**) **21**, **26**, **31**

(**17**) **22**, **27**, **32**

(**18**) **23**, **28**, **33**

(**19**) **24**, **29**, **34**

(**20**) **25**, **30**, **35**

Highlights

1. Novel library of mono- and disubstituted 2(5*H*)-furanone derivatives was successfully obtained.
2. Most active compound **12** displayed high selectivity towards malignant cell line A549.
3. Compound **12** induced G2 cell cycle arrest, suppressed colony formation and promoted caspase-independent cell death in A549 cell line.
4. Compound **12** showed slight synergetic effects with erlotinib against A549 cell line.