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2,4,5-Tris(alkoxyaryl)imidazoline derivatives as potent scaffold for novel p53-MDM2 interaction inhibitors: Design, synthesis, and biological evaluation

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ABSTRACT

Imidazoline-based small molecule inhibitors of p53-MDM2 interaction intended for the treatment of p53 wild-type tumors are the promising structures for design of anticancer drugs. Based on fragment approach we have investigated a key role of substituents in *cis*-imidazoline core for biological activity of nutlin family compounds. Although the necessity of the substituents in the phenyl rings of *cis*-imidazoline has been shown, there are no studies in which the replacements of a halogen by other substituents have been investigated. A series of simple *cis*-imidazoline derivatives containing halogen, hydroxy and alkoxy-substituents were synthesized. The biological activity of the compounds was studied using assays of cytotoxicity (MTT) and p53 level. It was found that the hydroxyl-derivatives were not cytotoxic whereas the alkoxy analogues were the same or more active as halogen-substituted compounds in cell viability test. The synthesized alkoxy derivatives induced an increase of p53 level and did not promote necrotic cell death in the concentration up to 40 μM.

p53-MDM2 system represents a unique class of protein–protein interaction (PPI) for target therapy in cancer studies.[1–3] p53 tumor suppressor is a principal mediator of cell cycle arrest, senescence, and apoptosis in response to a broad array of cellular damage.[4] In normal unstressed cells, p53 is a very unstable protein, the cellular level of which is low enough due to its degradation largely mediated by MDM2.[1] Through three inhibitory mechanisms, MDM2 functions as an effective antagonist of p53.[2]

Because MDM2 plays a key role in inhibition of the p53 tumor suppressor functions and downregulates p53 through direct PPI. MDM2 acts as an ubiquitin-protein ligase and targets p53 for proteasomal degradation. A blockage of the MDM2-p53 PPI releases p53 from MDM2, restoring the tumor suppressor functions of wild-type p53. Agents designed to block the MDM2-p53 interaction have a therapeutic potential for the treatment of various cancers retaining wild-type p53.[2] MDM2 binds p53 through its hydrophobic cleft and its blockage by small molecules can reactivate p53 functions and promotes apoptotic death of cancer cells.[5–8] A class of imidazoline compounds, termed nutlins, interacts specifically with the p53-binding pocket of MDM2 and inhibits the p53-MDM2 interaction.[9] Importantly, nutlin analogues have being tested in clinical trials for treatment of different cancers.[10] Nutlins' molecular structure (Fig. 1) is capable to bind the site of MDM2, which is responsible for interaction with *N*-terminal domain of p53.[11–15] The two *cis*-phenyl substituents directly insert into two pockets of the binding site (Trp23 and Leu26), whereas a third phenyl substituent indirectly reaches the third pocket (Phe19) by means of an *ortho*-isopropoxy or ethoxy group.[5] Filling of a small cavity of the Trp23 and Leu26 pockets usually with a halogen seems to be a critical feature of an efficient MDM2 inhibitor.[5,6] The fourth imidazoline substituent, the N-2-hydroxyethylpiperazine ring, does not penetrate the p53-binding cleft directly but instead covers the Phe19 pocket near the Met62 side chain of MDM2. This heterocyclic motif likely increases water solubility of a compound.[5]

Deconstruction of nutlins family into small fragments have shown that imidazoline fragment without substitution at N atom possess binding MDM2 activity (Fig. 1).[16] The necessity of halogen substituent and *cis*-configuration of aromatic rings at C4 and C5 position was of great importance.[5,15] Although the necessity of the substituents in the aromatic ring has been shown, there are no studies in which the replacement of a halogen in a nutlin molecule by another substituent

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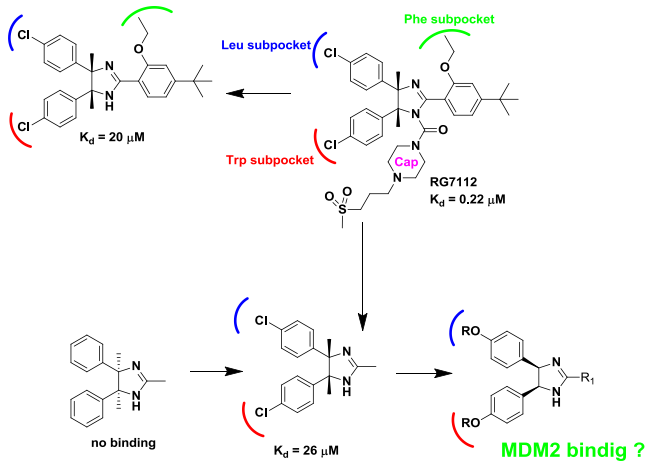


Fig. 1. MDM2 binding activity for Nutlin analog and its fragments and proposal halogen atom substitution.[16]

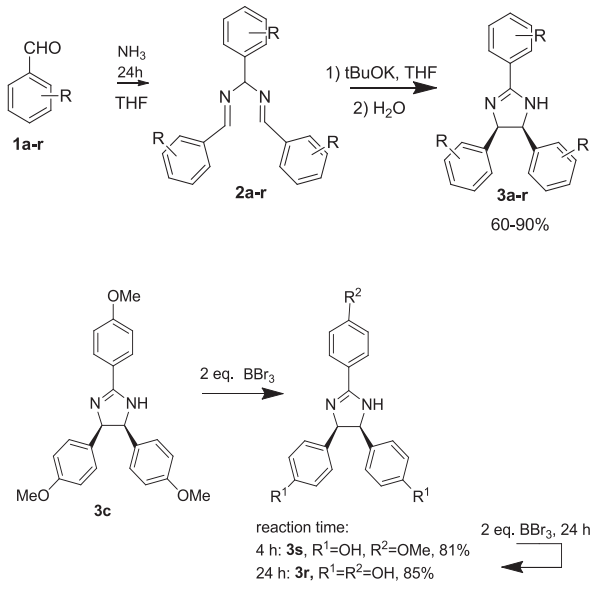
would be studied. The reason for this is the lower availability of other aryl-substituted *cis*-vicinal diamines, which are precursors in the synthesis of nutlins and their analogues. The aim of this study is to predict potential MDM2 binding and an inhibition of MDM2-p53 interaction of nutlin analogues containing other substituents in the aromatic cycle, such as alkoxy and hydroxy groups, by using of a simple *cis*-imidazoline fragment-based approach.

Synthetic approach was based on the reaction of aromatic aldehydes with ammonia solution, leading to synthesis of trimeric products – 1,3,5-trisaryl-2,4-diaza-1,4-pentadienes. The latter, in turn, produced *cis*-imidazolines by the action of potassium *t*-butoxide or another strong base in an aprotic solvent. The disrotatory closure of the pentadiene cycle led precisely to the cis product, which was indicated by the chemical shifts of the C—H protons of the imidazoline ring. For the cis structures, a signal of C—H protons of the imidazoline ring was about 5–5.5 ppm, while for the trans structures, a shift was observed at 4–5 ppm. It should be noted that during long-term treatment of the cis product with a strong base such as potassium *t*-butoxide the isomerization to the more stable *trans*-isomer occured.[17]

The cleavage of the methoxy groups to obtain the derivatives **3r–v** was carried out in presence of boron tribromide. We have found that the higher reactivity of aryloxy groups in position 4 and 5 of imidazoline ring vs. position 3 can be used to obtain selectively hydrolyzed compound **3s** in kinetic conditions (low excess of BBr_3 and short reaction time, Scheme 1).

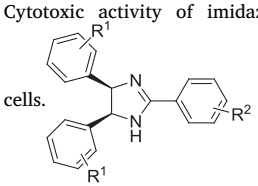
The cytotoxicity of the obtained imidazolines against lung carcinoma cell line A549 was evaluated using MTT assay. The cytotoxic activity of alkoxy substituted imidazolines was comparable with those of halogen-substituted compounds usually used as nutlin core (Fig. 1, Table 1). The absence of alkoxy group in aryl substituents in position 4,5 of imidazoline or its changing to hydroxy group led to dramatic loss of cytotoxicity at all.

To investigate the possible binding mode for this set of compounds, we selected the most active compound – **3c**, **3f**, **3j**, **3m** – to perform the molecular docking simulations.[18] The aim of this work was to find a new imidazoline core with prominent MDM2 binding activity and increased water solubility. The water solubility is one of the crucial points for effective drug design. The compounds **3p** and **3l** containing halogen groups demonstrating significantly less soluble in water then methoxy-containing imidazolines were not tested. The compounds **3c** and **3j** were tested to evaluate the role of substitution in the position 4 of aromatic ring. The compound **3m** was used as a reference because this compound had the same substitution in 4,5 position of the imidazoline core like the nutlins. However, the compound **3m** did not have additional groups increasing binding affinity to MDM2 so we compared



Scheme 1. Synthesis of new *cis*-imidazoline derivatives.

Table 1
Cytotoxic activity of imidazoline derivatives **3a–v** against A549 cancer cells.



Compound	R	A549 (IC50, μM)
3a	2-MeO (R^1 = R^2)	27.36 ± 0.79
3b	3-MeO (R^1 = R^2)	64.31 ± 1.04
3c	4-MeO (R^1 = R^2)	43.90 ± 1.87
3d	2,3-diMeO (R^1 = R^2)	16.18 ± 0.29
3e	2-EtO,3-MeO (R^1 = R^2)	24.26 ± 2.10
3f	2,4-diMeO (R^1 = R^2)	9.32 ± 0.47
3g	3,4-diMeO (R^1 = R^2)	84.69 ± 0.76
3h	2,5-diMeO (R^1 = R^2)	21.42 ± 1.07
3i	3,4,5-trisMeO (R^1 = R^2)	n.a.*
3j	4-EtO (R^1 = R^2)	13.26 ± 0.37
3k	3-MeO,4-EtO (R^1 = R^2)	67.49 ± 0.04
3l	2-Cl (R^1 = R^2)	10.68 ± 0.18
3m	4-Cl (R^1 = R^2)	20.25 ± 1.88
3n	2,4-diCl (R^1 = R^2)	164.41 ± 13.74
3o	3,4-diCl (R^1 = R^2)	13.72 ± 1.02
3p	4-Br (R^1 = R^2)	9.05 ± 0.20
3q	4-F (R^1 = R^2)	107.83 ± 4.65
3r	4-OH (R^1 = R^2)	n.a.*
3s	4-OH(R^1),4-MeO(R^2)	n.a.*
3t	3-OH (R^1 = R^2)	310.60 ± 16.67
3v	2,5-diOH (R^1 = R^2)	n.a.*
nutlin-3a		15.12[15]

*n.a. means the absence of inhibition activity in concentration range of 1.56–100 μM.

biological activities of "pure" methoxy and halogen-substituted imidazoline cores. The 3D structure of MDM2 for docking simulation was obtained from PDB (PDB id: 4HG7). The compounds successfully docked inside the same active binding site of MDM2 where p53 peptide binds with a energy in a range of −6 to −9 Kcal/mol. As shown in Fig. 2, the obtained results were similar to those reported for nutlin-3a.[15] The *cis*-methoxyphenyl ring directly inserted into two pockets of the binding site of MDM2 (Trp23 and Leu26) whereas the third phenyl substituent indirectly reached the third pocket (Phe19).

We analyzed the abilitiy of synthesized derivatives to inhibit MDM2-p53 interaction and stabilize p53. For this evaluation also

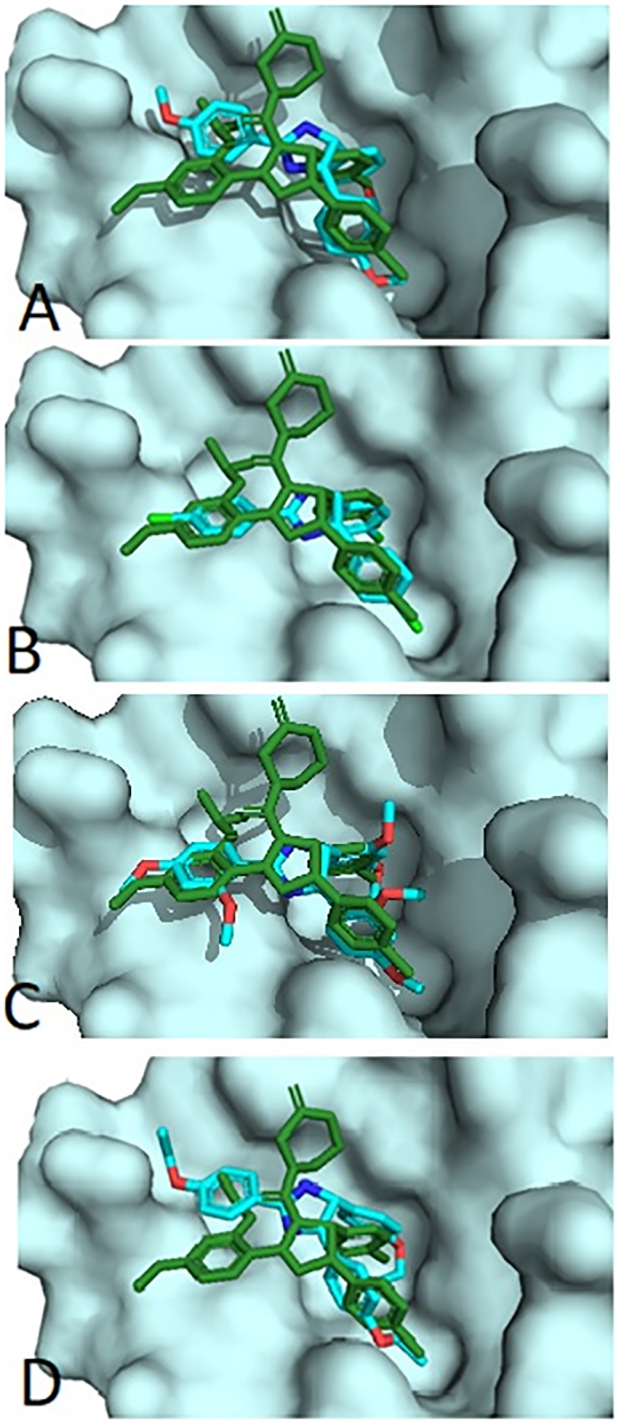


Fig. 2. Docked pose of (A) **3c**, (B) **3m**, (C) **3f**, (D) **3j** (blue stick) overlaid with Nutlin 3a (green) in p53 binding site of MDM2 protein.

compounds **3c**, **3f**, **3j, 3m** were selected. For study of biological activity of the compounds lung adenocarcinoma cell line A549 was chosen. Importantly, the basal level of p53 in A549 cells is low enough. A treatment with nutlins or their analogues has been shown to result in essential stabilization of p53 level in A549.[19] In contrast to A549, nutlin-mediated p53 stabilization was not been detected in many cell lines.[19] Moreover, nutlins and their analogues have been shown not to induce apoptosis alone in A549 cells. It happens only upon combination of nutlins with other agents, e.g. DNA-damaging drug cisplatin.[20,21] In many other cell lines nutlins induce apoptosis,[22,23] which leads to caspase- and calpain-dependent cleavage of p53 and a drop of its level

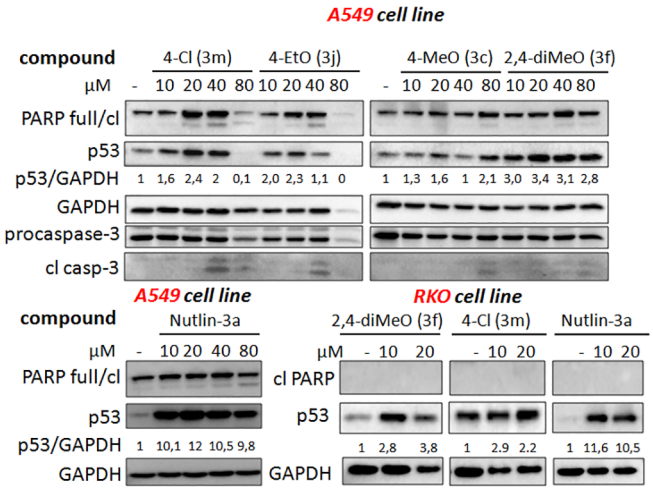


Fig. 3. The selected derivatives stabilized p53 level. Western Blot analysis of total cellular lysates from A549 or RKO cells upon treatment with indicated compounds. GAPDH was used as a loading control. Designations: PARP full/cl – full form and p89 fragment of PARP; GAPDH – glyceraldehyde 3-phosphate dehydrogenase; procasp-3 – procaspase-3; cl casp-3 – p19/17 fragments of caspase-3; p53/GAPDH – densitometric analysis of p53P bands normalized to GAPDH. Data from 3 biological replicates.

in the cell.[24–26] Consequently, if nutlins or their analogs promote apoptosis induction, decrease of p53 level interferes with nutlin-induced increase of this protein. Accordingly, for correct estimation of p53 stabilization we used cell line A549.

A549 cells were treated with the compounds **3c**, **3f**, **3j** and **3m** in concentrations of 10, 20, 40, and 80 μM. These concentrations were selected according to IC50. After treatment during 24 h cells were collected and p53 level was estimated using Western-blot (WB) approach (Fig. 3). The analysis confirmed that all compounds stabilized p53. 2,4-diMeO derivate **3f** at 20 μM demonstrated the best efficacy for p53 stabilization. Densitometric analysis of p53 bands normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) showed that the treatment with this compound led to 3.4-fold increase of p53 level compared to non-treated cells. For agents **3c**, **3j** and **3m** the most efficient concentration was also 20 μM, which increased p53 level for 1.6, 2.3 and 2.4 times, correspondingly (Fig. 3). Additionally, Nutlin-3a in concentration of 10, 20, 40, and 80 μM was tested as a positive control. According to WB analysis, the treatment with Nutlin-3a led to 10.1–12-fold increase of p53 level compared to non-treated cells A549. Considering the fact that the synthesized molecules don't comprise all parts of nutlins, the efficacy of 2,4-diMeO derivate **3f** was comparable to Nutlin-3a.

Additionally, colon carcinoma cell line RKO was used to confirm the efficacy of 2,4-diMeO derivate **3f**. According to WB analysis, the treatment of RKO cells with the most efficient compound **3f** in concentration of 10 and 20 μM increased p53 level for 2.8 and 3.8 times, correspondingly (Fig. 3). The treatment of RKO with Nutlin-3a in concentration of 10 and 20 μM led to 11.5–10.6-fold increase of p53 level compared to non-treated cells RKO. Taken together, we demonstrated that if cells (A549 and RKO) are sensitive to Nutlin-3a, the treatment of these cell lines with the most efficient 2,4-diMeO derivate also leads to pronounced increase of p53 level.

To study whether these compounds might induce apoptosis activation of death effector caspase-3 and cleavage of its substrate – PARP (poly(ADP-ribose) polymerase 1) – were analyzed. Importantly, Nutlin-3 in concentrations up to 20 μM was shown not to induce apoptosis alone in A549 cells.[20,21] According to WB analysis the compounds **3j** and **3m** in concentration of 40 μM induced a generation of caspase-3 p17/19 active fragment and subsequent cleavage of PARP (Fig. 3). The treatment with 80 μM of these agents led to essential cell death (Fig. 4)

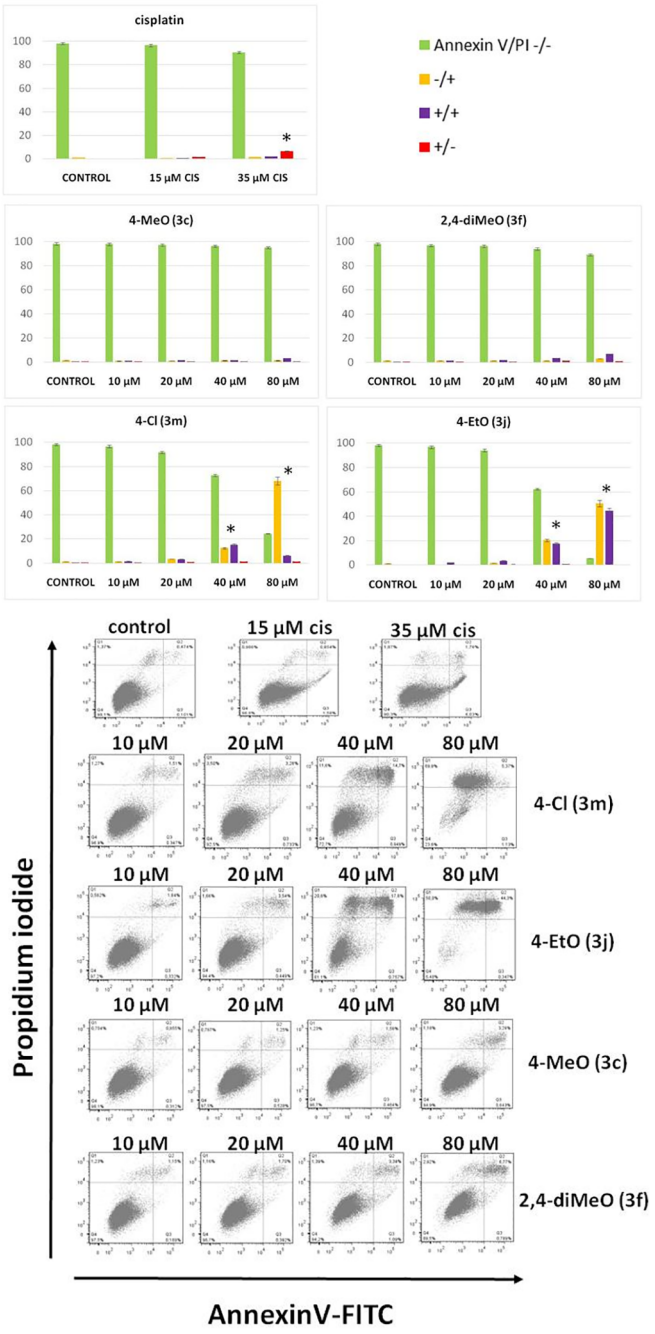


Fig. 4. Flow cytometry (FC) analysis of A549 cells treated with cisplatin, **3c**, **3f**, **3j** and **3m**. Cells were stained with the conjugate of Annexin V-FITC and Propidium iodide (PI). Designation for Annexin V/PI: −/−, viable cells; +/− apoptotic cells; +/+ late apoptotic cells; +/− necrotic cells. (A) FC data for control and treated cells. (B) Histograms of FC data for cells treated with the compounds at different concentrations. All experiments were performed at least three times. Results, if not otherwise stated, are presented as mean ± standard deviation (SD). *$p < 0.05$: significant difference compared to vehicle treated sample (Mann-Whitney *U* test).

and full degradation of cellular proteins (Fig. 3). At the same time, the treatment of cells with the compounds **3c** and **3f** led to week caspase-3 activation and PARP cleavage even at concentration of 80 μM. Taken together, the 4-MeO- and 2,4-diMeO-derivatives **3c** and **3f** did not induce apoptotic cell death in concentration up to 40 μM. In contrast, the administration of 4-Cl- and 4-EtO-derivatives **3j** and **3m** at 40 μM resulted in measurable activation of caspases and subsequent apoptosis. Importantly, the treatment with 80 μM of **3j** and **3m** did not increase p53 level but led to more pronounced cell death. Accordingly, these compounds induced cell death due to non-specific toxicity rather than p53 stabilization. Moreover, the administration of 2,4-diMeO-derivate **3f** led to the most efficient stabilization of p53 level but not to apoptosis.

Collectively, our findings demonstrate that the selected compounds are able to inhibit MDM2-p53 interaction and promote p53 stabilization. According to WB analysis the synthesized compounds at concentration of 20 μM promoted p53 stabilization to different extents.

To evaluate apoptotic and necrotic cell death A549 cells were treated with the compounds **3c**, **3f**, **3j** and **3m** in concentration of 10, 20, 40 and 80 μM for 24 h. Then, the cells were stained with Annexin V – FITC in combination with propidium iodide (PI) and analyzed on a flow cytometer.

Double staining (Annexin V/PI +/+) was used to evaluate the population of late apoptotic and secondary necrotic cells. Early apoptotic cells were stained with annexin V-FITC only (Annexin V/PI +/−), and necrotic cells were stained with PI only (Annexin V/PI −/+) (Fig. 4). As a positive control for triggering apoptosis the treatments with 15 and 35 μM of cisplatin were used.

Cell death analysis using Annexin V-FITC/PI staining revealed that the cisplatin treatment led to an increase of early and late apoptotic populations – Annexin V/PI+/− and Annexin V/PI+/+, correspondingly. In contrast to cisplatin, 4-MeO- and 2,4-diMeO- derivatives **3c** and **3f** in concentrations up to 40 μM did not increase apoptotic or necrotic populations (Fig. 4). The treatment with 80 μM of these compounds only slightly enhanced Annexin V-FITC/PI double staining of A549 cells. Obtained data were in accordance with WB analysis of caspase-3 and PARP cleavage (Fig. 3). 4-Cl- and 4-EtO-derivatives **3j** and **3m** at 40 and 80 μM induced essential accumulation of necrotic PI-positive cells up to 70% (Fig. 4). This was consistent with WB-detected prominent cleavage of cellular proteins, including PARP and caspase-3. This fact confirmed non-specific toxicity of 4-Cl- and 4-EtO-derivatives **3j** and **3m** in concentrations higher than 40 μM.

Altogether, these data indicated that 4-MeO- and 2,4-diMeO- derivatives **3c** and **3f** did not induce necrotic cell death in concentration up to 80 μM and were able to stabilize p53. In contrast, 4-Cl- and 4-EtO-derivatives **3j** and **3m** promoted necrotic cell death and were less efficient for p53 stabilization. Additionally, the necessity of methoxy-group in the position 4 of imidazoline core was investigated. A549 cells were treated with the compounds **3d** and **3h** in concentration of 10 and 20 μM. These compounds were isomers of the derivatives **3f** and **3c** but didn't have *para*-substituent in phenyl. The WB analysis confirmed that both compounds demonstrated the lack of p53 stabilization that suggested the absence of MDM2-p53 interaction blocking activity (see Fig. S1).

New *cis*-imidazoline derivatives containing methoxy and hydroxy groups were synthesized using the reaction of aromatic aldehydes with ammonia. It was shown that the alkoxy substituents of obtained arylimidazolines had different reactivity and alkoxyaryl in position 4,5 of imidazoline ring could be hydrolyzed separately from alkoxy group of aryl in position 2. No one of the hydroxy-derivatives including partially hydrolyzed showed antiproliferative activity whereas alkoxy-containing imidazolines were cytotoxic at a micromolar range of concentrations. This fact shows the importance of the 4-alkoxyaryl substituent in positions 4 and 5 of imidazoline ring to retain the cytotoxic activity comparable to 4-halogenaryl substituted imidazoline, which is usually used as nutlin core and has good water solubility. The selected compounds demonstrated biological activity, promoting stabilization of p53 level in lung adenocarcinoma cells A549.

Despite the fact that synthesized molecules did not comprise all parts of nutlins, these compounds were able to inhibit MDM2-p53 interaction and increased p53 level in cells. 2,4-diMeO derivate **3f** has been shown to possess the best efficacy for p53 stabilization. The treatment with this compound led to 3.5–3.8-fold increase of p53 level. This compound only slightly stimulated apoptosis and did not induce

necrotic death in A549 cells. These results are in accordance with other reports demonstrating that Nutlin-3 is not able to induce apoptosis alone in A549 cells and can do that only in combination with other agents. At the same time the 4-Cl- and 4-EtO-derivatives **3j** and **3m** showed lower activity for p53 stabilization and induced necrotic cell death. According to the flow cytometry, these compounds stimulated necrosis in concentrations higher than 20 μM.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2019.06.007.

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