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Computer-aided search for 5-arylideneimidazolone anticancer agents able to overcome ABCB1-based multidrug resistance

Aneta Kaczor ^[a], Nikolett Szemerédi ^[b], Dr. Katarzyna Kucwaj-Brysz ^[a], Dr. Monika Dąbrowska ^[c], Dr. Małgorzata Starek ^[c], Dr. Gniewomir Latacz ^[a], Dr. Gabriella Spengler^[b], and Prof. Jadwiga Handzlik ^{*[a]}

- [a] A. Kaczor, Dr. K. Kucwaj-Brysz, Dr. G. Latacz, Prof. J. Handzlik
Department of Technology and Biotechnology of Drugs
Faculty of Pharmacy, Jagiellonian University Medical College
9 Medyczna Street, 30-688 Kraków, Poland
E-mail: aneta.kaczor@doctoral.uj.edu.pl ; katarzyna.kucwaj@uj.edu.pl ; gniewomir.latacz@uj.edu.pl ; j.handzlik@uj.edu.pl
- [b] N. Szemerédi, Dr. G. Spengler
Department of Medical Microbiology and Immunobiology
Faculty of Medicine, University of Szeged
Dóm tér 10, H-6720 Szeged, Hungary
E-mail: szemeredi.nikoletta@med.u-szeged.hu ; spengler.gabriella@med.u-szeged.hu
- [c] Dr. M. Dąbrowska, Dr. M. Starek
Department of Inorganic Chemistry
Faculty of Pharmacy, Jagiellonian University Medical College
9 Medyczna Street, 30-688 Kraków, Poland
E-mail: monika.1.dabrowska@uj.edu.pl ; m.starek@uj.edu.pl

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Abstract: ABCB1 modulation is an interesting strategy in search for new anticancer agents overcoming multidrug resistance (MDR). Hence, 17 new 5-arylideneimidazolones containing amine moiety, as potential ABCB1 inhibitors was designed, synthesized, and investigated. The series was tested in both, parental (PAR) and multidrug resistant (MDR) overproducing ABCB1, T-lymphoma cancer cells using cytotoxicity assay. The ABCB1 modulating activity was examined in the rhodamine 123 accumulation test, followed by Pgp-Glo™ Assay to determine an influence of most active compounds on ATPase. Lipophilic properties were assessed both, in silico and experimentally (RP-TLC). Pharmacophore-based molecular modelling towards ABCB1 modulation was performed. The studies allowed to find anticancer agents (p-fluorobenzylidene derivatives) more potent than doxorubicin, with highly selective action on MDR T-lymphoma cells (selectivity index > 40). Most of the investigated compounds showed ABCB1-modulating action, especially, two 5-benzyloxybenzylidene derivatives displayed activity nearly as strong as tariquidar.

Introduction

Multidrug resistance (MDR) is the significant problem in the treatment of cancer [1]. For most patients, who suffer from metastatic cancer, treatment fails because of MDR [1,2]. Due to this substantial situation, a search for new therapeutic solutions to overcome cancer MDR is of great importance for current medicinal science. One of proposed approaches are “adjuvants” for anticancer drugs, which are able to block at least one of the mechanisms of MDR. In this context, many studies have been carried out in order to find compounds capable to modulate transport proteins representing one of the main mechanisms of MDR in cancer cells [1,3,4]. Among them, the efflux pumps that belong to ATPase binding cassette (ABC) family are of great scientific interest [1]. In particular, ABCB1 transporter, often

named glycoprotein P (Pgp), is widely described target that has complex structure with many drug-binding domains, and consequently, wide variety of substrates [1]. Various assays have indicated significant role of this efflux pump in cancer MDR due to the potency to expel many, structurally unrelated, anti-cancer drugs. Furthermore, the overexpression of this pump in clinical MDR cancer cells has been confirmed [1,5,6]. Thus, searching for ABCB1 modulators has been a topic of cancer medicinal chemistry for decades. As results, various active structures, which can be divided into four generations, have been found. Compounds, which belong to the first and second generations of ABCB1 modulators, were found among known drugs, e.g. verapamil or cyclosporine A [7]. However, these generations indicate action on other targets, usually more potent than that on ABCB1, which limits their application as “adjuvants” in cancer treatment [1,7]. The third generation of ABCB1 inhibitors, e.g. tariquidar or zosuquidar, were significantly more potent than previous generations [6]. Unfortunately, these compounds are not enough safe for therapeutic usage due to various toxic effects [3,6]. One of the explanations is their toxicity to healthy human cells due to the presence of ABCB1 efflux pump in both, normal and tumor cells [1,3]. Dash et al. pointed out ADME properties as a main drawback of those compounds [8]. Nowadays, there are studies conducted with natural products e.g. chalcones, flavonols, flavones, which were assigned as the fourth generation [9-11]. Despite higher safety of the new compounds, various disadvantages associated with them have been reported. Currently, there are studies focused on synthetic modifications of natural products in order to boost pharmacokinetic parameters [10-13].

At the continuous absence of ABCB1-targeted “adjuvants” in pharmaceutical market, and taking into account their potential advantages for the cancer therapy development, the search for ABCB1 modulators is still a great challenge of pharmaceutical

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science, involving various approaches of medicinal chemistry. Thus, Cianchetta et al. pointed out that pharmacophore model rather than physicochemical properties prediction allowed for recognition of ABCB1 substrates among series of designed chemical compounds [14]. They presented one model for all substrates, which showed some similarities to other pharmacophore models [14-16]. However, various studies have indicated differences between pharmacophore features, depending on structure of the compounds that were used for their hypotheses [14,17]. For instance, the pharmacophore postulated by Reyes et al. does not possess basic tertiary amine, which was presented in other models [17,18]. Due to that, it seems, that pharmacophore models should be created separately for each chemically-related group of designed and tested ABCB1 modulators.

On the other hand, physicochemical properties seem to play crucial role in the ABCB1 modulating potency. Various lines of evidence pointed out shape and size as important feature [14,19], while lipophilicity is the most frequently considered property. Hence, Cianchetta et al. [14] indicated a weak correlation of logP in their model, whereas Wiese and Pajeva [18] emphasized high lipophilicity as desirable feature of ABCB1 modulators. Moreover, Jain et al., based on their work on sipholane triterpenoids (**1**, Figure 1) [20], accented that strong ABCB1 modulators should possess logP higher than 2.92. In contrast, poor correlation was indicated between activity and logP for chalcone derivatives (**2**, Figure 1) [21]. Although the aforementioned results does not provide coherent information about the role of lipophilicity for ABCB1 modulators, they indicate that the assessment of this property should be an integral part of initial search for ABCB1 modulators in any new chemical family.

Taking into account both, the need to find effective and pharmacologically safe ABCB1 modulators that finally would get pharmaceutical market and the growing interest of modern medicinal chemistry in multi-target drugs, it is especially promising to search for new anticancer agents, selective towards MDR cancer cells, with simultaneous MDR-reversal properties.

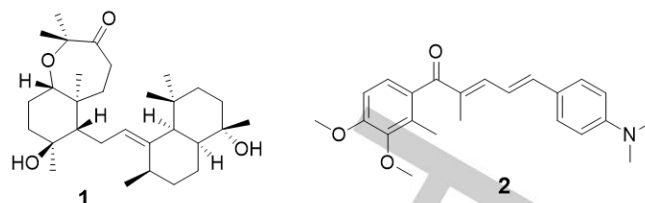


Figure 1. Examples of potential ABCB1 modulators from the group of sipholane triterpenoids (**1**) and chalcones (**2**) [20,21].

Our previous studies indicated that some of imidazolone and hydantoin derivatives were able to modulate the efflux pump ABCB1 [22-24]. The specific activity in the ethidium bromide assay was found for the amine-free hydantoin with benzyloxybenzylidene moiety at position 5 (**3**, Figure 2) [23]. In particular, imidazolones with an amine group at position 2 or 2 and 3, demonstrated the potential ABCB1 modulating activity (**4** and **5**, Figure 2) stronger than verapamil [24]. Previous studies have provided useful conclusions coming from performed SAR analysis. Thus, the following traits for imidazolones able to modulate ABCB1 have been postulated: (i) the aromatic moiety; (ii) the positive ionizable center; (iii) the hydrophilic moiety, (iv) the alkyl linker and (v) the hydrogen bond acceptor [22]. However, the anticancer cytotoxicity of those series was usually negligible or moderate in the best case [22-24].

Although our previous search for the imidazolone-derived ABCB1 modulators was comprehensive, two important aspects have been missed so far, *i.e.* a comparison of the activities between those compounds and strong inhibitors of third generation as well as a pharmacophore model for 5-arylideneimidazolones. Above all, previous pharmacomodulation in the imidazolone group was focused on a single ABCB1-modulating action, and their anti-tumor effects did not clearly differentiate between MDR- and reference cancer cells (selectivity index, SI < 1) [24].

For the aforementioned reasons, searching for more potent ABCB1-modulators, with MDR-selective anticancer action among further imidazolone derivatives was needed. In this context, a general structure for new imidazolones (**6**, Figure 2) as combination of fragments coming from previously found active lead structures **3-5** [22-24], has been proposed for a further exploration.

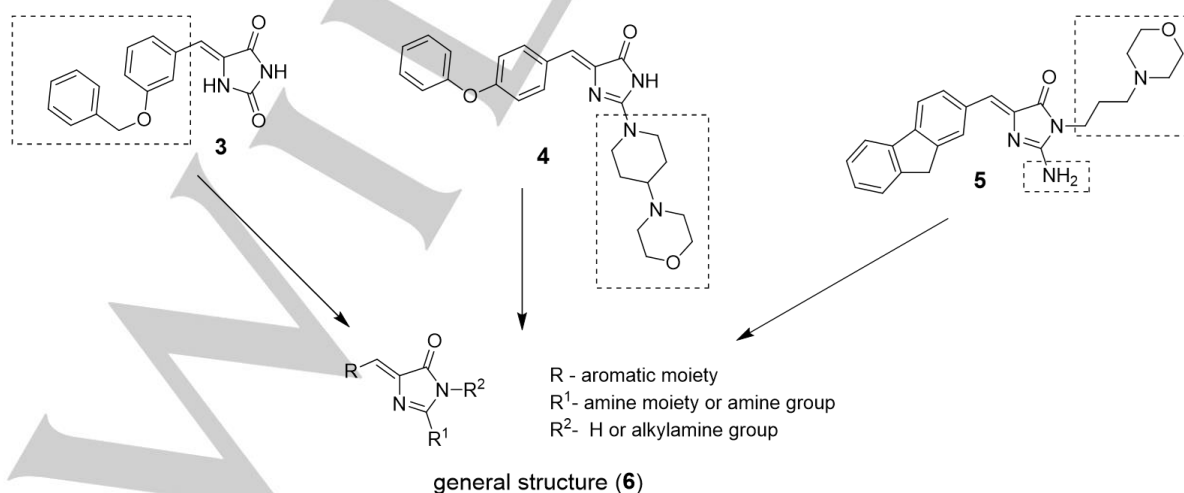
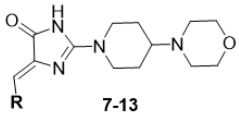


Figure 2. Previously found active ABCB1 modulators in the group of imidazolones (**3-5**) and the general structure of series explored in current studies (**6**) [23,24].

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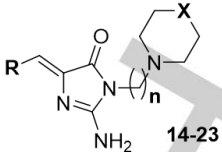
Within this study, a series of 17 new 5-arylideneimidazolone derivatives (**7-23**), covering the general structure **6**, was designed, synthesized and investigated. The series was divided into two groups depending on position of the amine moiety, *i.e.* the group A (**7-13**, Table 1) with the amine fragment only in position 2 of the imidazolone ring and the group B with amines at both, 2 and 3, positions (**14-23**, Table 2). The compounds were examined on their cancer cell cytotoxic effects, followed by an assessment of ABCB1 efflux pump modulating properties using *in vitro* assays. The ATPase substrate property for selected compounds was determined. Based on the obtained results, molecular modelling, in order to elaborate new pharmacophore hypothesis useful for imidazolone ABCB1 modulators, was performed. In term to verify an influence of physicochemical properties on the considered biological actions, the lipophilicity tests using both, experimental and *in silico*, methods were conducted for the whole series (**7-23**). Finally, SAR analysis was performed and discussed.

Table 1. Structures of the investigated compounds from group A (**7-13**).



Cpd	R	Cpd	R
7		11	
8		12	
9		13	
10			

Table 2. Structures of the investigated compounds from group B (**14-23**).



Cpd	R	n	X
14		3	O
15		3	N-CH ₃
16		3	N-C ₆ H ₅
17		2	O
18		3	O
19		3	N-CH ₃
20		3	O
21		3	N-CH ₃
22		3	O
23		3	N-CH ₃

Results and Discussion

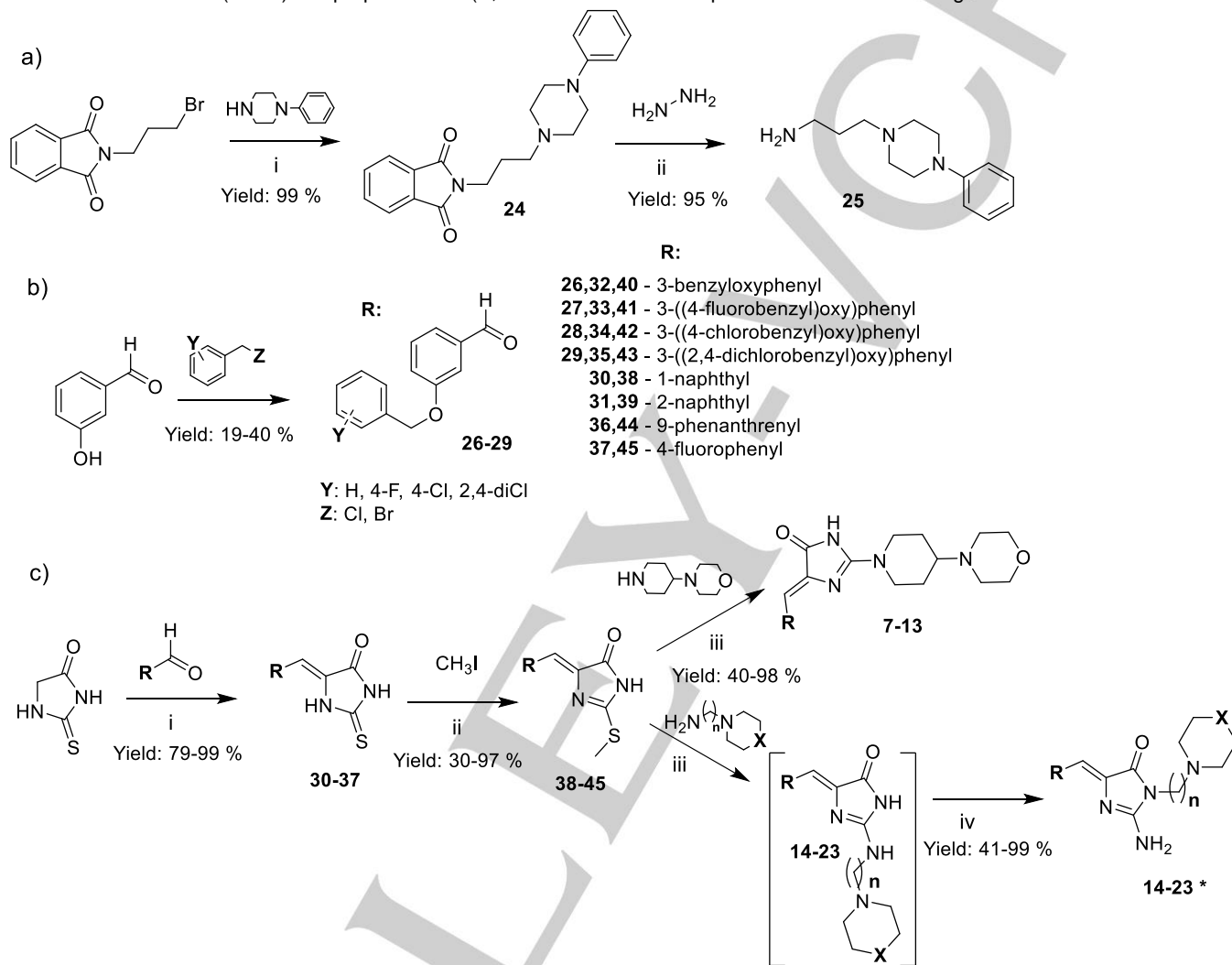
Chemical synthesis

Final products (**7-23**) were obtained in the 3-5-step synthetic pathways (Scheme 1). Methods of synthesis for intermediates **30**, **31**, **36**, **38**, **39**, **44** and the final product **7** were described previously [25,26]. First (Scheme 1a), two-step Gabriel synthesis was performed, *i.e.*: (i) the N-alkylation of phenylpiperazine with the bromopropyl phthalimide to give intermediate **24**, followed by (ii) hydrazinolysis of **24** to obtain the primary amine derivative of phenylpiperazine (**25**), useful as an intermediate for further synthesis. As the next group of intermediates, the 3-benzyloxybenzaldehydes (**26-29**) were obtained via O-alkylation of 3-hydroxybenzaldehyde with appropriate benzyl chlorides or bromide using two-phase alkylation conditions (Scheme 1b). Then, Knoevenagel

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condensation of aldehydes (**26-29** or the commercially available ones) with 2-thiohydantoin was carried out (i, Scheme 1c), which gave the 5-arylideneimidazolones (**30-37**) in Z-configuration, preferable for this kind of syntheses according to previous crystallographic studies [25, 26]. In the next step (ii, Scheme 1c), the intermediates **30-37** were S-methylated with iodomethane to give the S-methyl-5-arylideneimidazolones (**38-45**). In the last step, reactions between intermediates (**38-45**) and proper amines (iii, Scheme

1c) gave the final products **7-23**. In case of the reactions with primary amines, Dimroth rearrangement (iv, Scheme 1c) for compounds **14-23** was assumed, based on previous crystallographic studies and NMR results [25]. General mechanism of Dimroth rearrangement in this group was previously published [25], while example from current studies is provided in Supporting Information (Scheme S1). Products **7-23** were converted into hydrochloride salts by saturation of their post-reaction mixture with gaseous HCl.



Scheme 1. Synthesis of compounds **7-45**. a) Synthesis of 3-(4-phenylpiperazin-1-yl)propan-1-amine: (i) 1-phenylpiperazine, K_2CO_3 , TEBA, acetone, reflux, 5 h, rt, 72 h; (ii) hydrazine, ethanol anhydrous, reflux, 6 h, rt, 72 h. b) Synthesis of the 3-benzyloxybenzaldehyde derivatives (**26-29**): (un)substituted benzyl halide, K_2CO_3 , ethanol, reflux, 6 h. c) Synthesis of final compounds **7-23**: (i) appropriate aromatic aldehyde, CH_3COONa , CH_3COOH , reflux, 5-7 h; (ii) CH_3I , C_2H_5ONa , rt, 24 h; (iii) appropriate amine, 125°C, 15 min, ethanol, reflux, 5-7 h, rt, 20 h, saturation with gaseous HCl; (iv) Dimroth rearrangement, saturation with gaseous HCl. * Dimroth rearrangement was confirmed by crystallographic studies for products of reactions between S-methyl imidazolones and the primary amines terminated and has been assumed for compounds **14-23** based on the structural analogy and NMR results.

Cytotoxicity assays

Prior to estimate an impact on ABCB1 for synthesized 2-amine-5-arylideneimidazolones (**7-23**), their cytotoxicity was examined using MTT assays [27]. The assays were performed in two mouse T-lymphoma cell lines: the chemosensitive parental (PAR) and the multidrug resistant (MDR) ones. The

MDR subline was acquired through transfection with human *MDR1* (*ABCB1*) gene. The cytotoxic effects were expressed with IC_{50} values. The IC_{50} values for PAR cells were divided by those for MDR cells to calculate selectivity index (SI, Table 3). Almost all of tested compounds (**7-23**) had moderate cytotoxicity ($IC_{50} > 20 \mu M$) in PAR cells. Only compound **23** showed stronger ($IC_{50} = 14.89 \mu M$) cytotoxic effect.

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Furthermore, this compound displayed even stronger activity ($IC_{50} = 8.80 \mu\text{M}$) in the case of MDR cells.

The most potent action in MDR cells was observed for compounds **14** and **15** with IC_{50} equal to 2.37 or 2.15 μM , respectively. Interestingly, these compounds were not cytotoxic towards PAR cells ($IC_{50} > 100 \mu\text{M}$), thus demonstrating highly selective action ($SI > 40$). In order to compare the selectivity of the series (**7-23**), the $SI = 1.5$ was established as a breaking point. Hence, $SI > 1.5$ was considered as an indicator of a selective anticancer agent. Based on this classification, compounds **9**, **12**, **14**, **15** and **23** were selective derivatives with a SI of > 2.9 , 1.87 , > 42.19 , > 46.51 , and 1.69 , respectively.

Table 3. Cytotoxicity of 5-arylideneimidazolones (**7-23**) in PAR and MDR mouse T-lymphoma cell lines.

Cpd	Mouse T-lymphoma cell line				SI
	PAR		MDR		
	IC_{50} (μM)	SD \pm	IC_{50} (μM)	SD \pm	
7	79.72	3.02	> 100	-	≤ 0.80
8	> 100	-	70.54	0.056	> 1.42
9	> 100	-	34.48	0.4	> 2.90
10	45.42	3.36	40.19	2.77	1.13
11	44.45	1.46	50.39	0.93	0.88
12	33.20	0.89	17.77	1.02	1.87
13	>100	-	>100	-	> 1.00
14	>100	-	<u>2.37</u>	0.11	<u>> 42.19</u>
15	>100	-	<u>2.15</u>	0.03	<u>> 46.51</u>
16	32.11	1.83	31.38	2.6	1.02
17	97.57	0.29	97.90	0.45	1.00
18	46.56	1.39	41.93	0.77	1.11
19	22.02	0.95	20.7	0.39	1.06
20	30.50	4.04	24.53	0.33	1.24
21	26.96	1.49	35.92	1.79	0.75
22	34.53	0.29	32.85	0.78	1.05
23	14.89	0.47	8.80	0.67	1.69
DOX	0.37	0.03	3.5	0.1	
DMSO	>2 V/V%	-	>2 V/V%	-	

PAR, parental T-lymphoma cells; MDR, multidrug-resistant T-lymphoma cells overproducing efflux pump ABCB1; SD, standard deviation calculated from four repetitions; SI, selectivity index; DOX, doxorubicin; starting concentration of tested compounds in the MTT assay: 100 μM . The best cytotoxicity and selectivity results underlined.

Rhodamine 123 accumulation assay

An ability of the 2-amine-5-arylideneimidazolones (**7-23**) to affect ABCB1 efflux pump was tested using rhodamine 123 accumulation assay. It was determined in both aforementioned, PAR and MDR, mouse T-lymphoma cell lines. Results of this assay were obtained using flow cytometry. This method measures the intracellular accumulation of rhodamine 123, which is a fluorescent substrate of the ABCB1 efflux pump. The data obtained were calculated and presented as a measure of efflux pump modulatory properties, named the fluorescence activity ratio (FAR). Tariquidar, the highly potent

representative of third generation ABCB1 inhibitors, was chosen as reference. The tested compounds (**7-23**) were divided into two separate experiments, which caused elicitation of various tariquidar FAR. Due to that, results were also expressed using FAR quotient (% of tariquidar FAR) to enable comparison of the action in the whole series with respect to that of tariquidar, but used in the significantly lower concentration.

Nine out of seventeen tested 5-arylideneimidazolones (**9**, **10**, **12**, **13**, **19-23**) showed activity of ABCB1 modulators (FAR = 3.095 - 30.860) at 20 μM concentration (Table 4). Compound **20** demonstrated a moderate activity (FAR = 11.331), while two others (**12** and **22**) displayed strong activity with FAR values 28.269 and 30.860, respectively. These two compounds (**12**, **22**) were nearly as effective as tariquidar (FAR quotient = 87.591 % or 95.620 %, respectively).

Table 4. Results of rhodamine 123 accumulation assay for 2-amine-5-arylideneimidazolones (**7-23**) tested in MDR mouse T-lymphoma cell lines.

Cpd	Results of Rhodamine 123 Accumulation			
	Concentration 2 μM		Concentration 20 μM	
	FAR	FAR Quotient (%)	FAR	FAR Quotient (%)
7 ¹	1.105	3.423	1.972	6.109
8 ¹	1.225	3.796	1.783	5.526
9 ¹	2.031	6.292	4.452	13.796
10 ²	1.617	4.325	3.214	8.599
11 ²	2.090	5.592	1.462	3.911
12 ¹	15.147	46.934	<u>28.269</u>	<u>87.591</u>
13 ²	1.952	5.223	5.976	15.987
14 ²	1.148	3.070	1.029	2.752
15 ²	1.536	4.108	1.276	3.414
16 ²	0.490	1.312	0.593	1.586
17 ²	0.357	0.955	0.495	1.325
18 ²	0.383	1.025	0.662	1.771
19 ¹	1.362	4.219	3.910	12.117
20 ¹	2.224	6.891	11.331	35.109
21 ²	1.521	4.070	3.095	8.280
22 ¹	15.807	48.978	<u>30.860</u>	<u>95.620</u>
23 ¹	2.120	6.569	6.360	19.708
TAR*	-	-	32.273 ^{1/} 37.381 ²	100.000
DMSO	0.678 ^{1/} 0.233 ² (V/V%)	2.102 ^{1/} 0.624 ²	-	-

Cpd, compound; TAR*, Tariquidar, tested in 0.2 μM concentration; DMSO, dimethyl sulfoxide; FAR, fluorescence activity ratio; FAR Quotient: compounds' FAR related to FAR of 0.2 μM TAR; ^{1,2} Compounds were tested in two groups with respect to different FAR values for TAR and DMSO. The two highest FAR and FAR quotients underlined.

Studies *in vitro* on ABCB1 modulation mechanisms

The most active 5-arylideneimidazolones in rhodamine 123 accumulation assay (**12**, **20**, and **22**) were chosen for the determination of potential molecular mechanisms of action in the assay *in vitro*. Thus, the luminescence Pgp-Glo™ Assay was carried out based on already described methods and protocols [24, 28-30]. This assay analyzes consumption of

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energy, necessary for efflux, which is obtained from ATP hydrolysis. The consumption of ATP is observed as decrease in luminescence. Basal activity, which demonstrates the ABCB1 basal ATP consumption, is considered as a difference between the luminescent signal of two samples: (i) treated with the selective and strong (100 %) ABCB1 inhibitor, Na_3VO_4 , and (ii) the luminescence of untreated ABCB1 samples. Hence, stimulators/substrates cause a statistically significant increase in the basal activity, while inhibitors give results below 100 % of the basal activity.

Compounds **12**, **20**, and **22** were tested in 100 μM concentration. Verapamil as reference, which possesses the substrate/ATPase stimulatory mode of action, at 200 μM was used. As a negative control, caffeine (inactive toward the ABCB1 transporter) was selected. Results are shown in Figure 3.

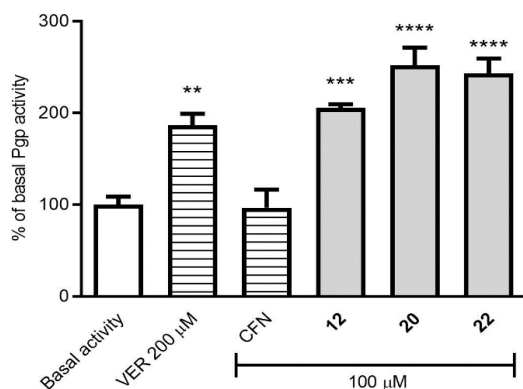


Figure 3. The effect of various compounds on ABCB1 basal activity as follows: VER, verapamil, the ABCB1 substrate (200 μM); CFN, caffeine, ABCB1-negative compound (100 μM); and 5-arylideneimidazolone derivatives **12**, **20**, and **22** (100 μM). ABCB1 substrates give results above 100% due to stimulation of basal activity. Results are presented as the mean \pm SD from 3 repetitions. One-way ANOVA was used for evaluation of statistical significance, and was followed by Bonferroni's comparison test (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ compared to the basal activity).

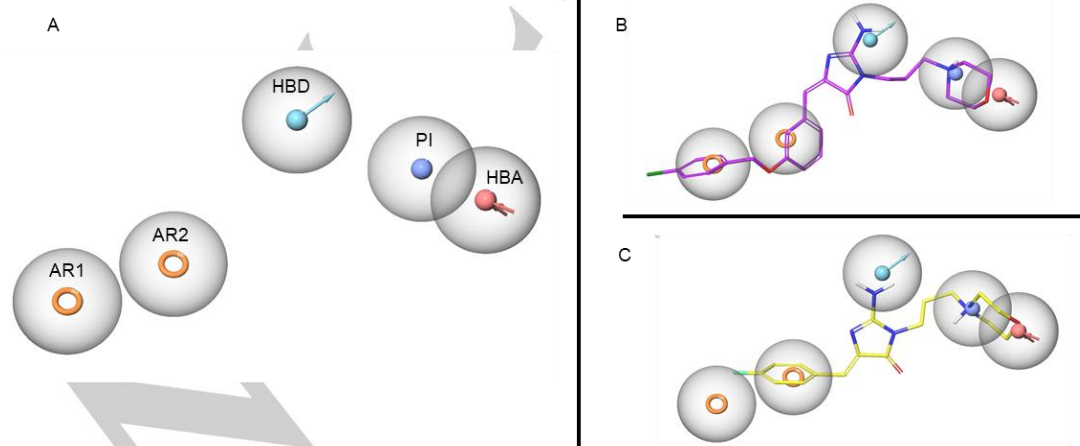


Figure 4. (A) The proposed pharmacophore hypothesis for MDR-reversing activity among 5-arylideneimidazolones (AR – aromatic ring; HBD – hydrogen bond donor; PI – protonable nitrogen atom; HBA – hydrogen bond acceptor; grey sphere – tolerance area $\pm 2\text{\AA}$); (B) the example of highly active compound **22** mapped to pharmacophore model; (C) the example of inactive compound **14** mapped to pharmacophore model.

All tested compounds (**12**, **20**, and **22**) caused the statistically significant ($p < 0.001$ or $p < 0.0001$) increase in basal activity, higher than that of verapamil. Thus, none of the three compounds displayed properties of ATPase inhibitor but, in similarity to verapamil, they can be considered substrates stimulating ATPase with different potency. The higher potency was observed for compounds representing group B (**20** and **22**), while the member of group A (**12**) was less active and only slightly more potent than verapamil.

In this context, the mechanism of ABCB1 modulation observed in the rhodamine 123 accumulation assay for compounds **12**, **20**, and **22** (Table 4) was not associated with the pump inactivation via ATPase inhibition. These results are in compilations with previous ones obtained for imidazolones and hydantoines [24, 27]. It suggests a competition with the dye-substrate towards ABCB1 binding site as the most probable mechanism of the pump modulating action for compounds **12**, **20** and **22**.

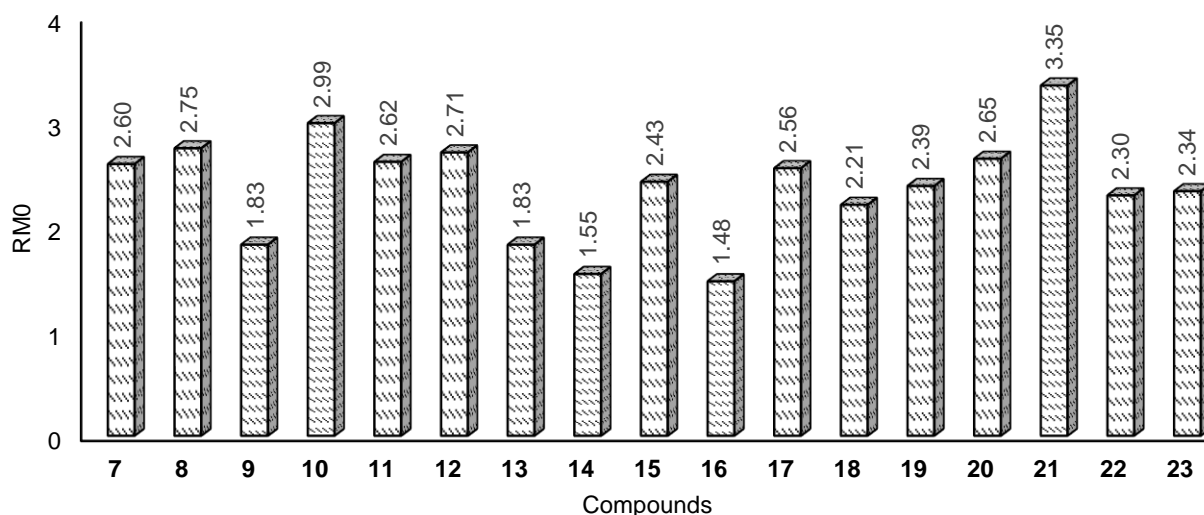
Pharmacophore model of ABCB1 modulator

Pharmacophore modelling was used in order to highlight the common structural features of the compounds with significant capacity to inhibit the MDR-efflux pump. The set of actives used to develop appropriate hypotheses consisted of compounds **12**, **20**, **22**, and lead structures **4** and **5**, whereas set of inactives included compounds **7-11**, **13-19**, **21**, and **23** (Figure 2, Table 1 and 2) [24]. The resulted pharmacophore model was developed using Phase (Schrödinger Software) [31] and postulated five structural features, *i.e.*: two aromatic rings (AR1 and AR2), hydrogen bond donor (HBD), protonable nitrogen atom (PI) and hydrogen bond acceptor (HBA, Figure 4A). All the distances between the particular structural features are presented separately in Table 5, in order to maintain the clarity of Figure 4A. Overlapping the structures of highly active compound **22** (Figure 4B) and inactive compound **14** (Figure 4C) into the hypothesis showed the necessity of presence of two aromatic rings instead of one ring.

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Table 5. The distances between structural features of the presented pharmacophore hypothesis.

Symbols of features	Distance [Å]
AR1-AR2	4.19
AR1-HBD	10.81
AR1-PI	13.93
AR1-HBA	15.73
HBD-PI	5.06
HBD-HBA	7.48
HBD-AR2	7.22
AR2-PI	9.84
AR2-HBA	11.77
PI-HBA	2.87

**Figure 5.** Lipophilicity (R_{M0}) of tested 2-amine-5-arylideneimidazolones (**7-23**) determined experimentally using RP-TLC method.

The 2-amine-5-arylideneimidazolones (**7-23**) displayed R_{M0} values between 1.48 and 3.35. Lipophilicity in both groups (A and B) was variable and no significant advantage was found for any group. Group B (**14-23**) consists of compounds with both, minimal (**16**) and maximal (**21**), values. In the case of group A (**7-13**), results for compounds with two fused rings (**7** and **8**) were quite similar to each other (2.60 and 2.75, respectively). The opposite situation could be seen for the unfused ring compounds ($R_{M0} = 1.83-2.99$). Interestingly, compound **13**, with the phenanthrene moiety at position 5, demonstrated rather low lipophilicity ($R_{M0} = 1.83$) in comparison to other representatives. An analysis of the results for imidazolones from group B with the same aromatic moiety, indicates that compounds with *N*-methylpiperazine fragment were more lipophilic than those with the corresponding morpholine moiety (**15** vs **14**, **19** vs **18**, **21** vs **20** and **23** vs **22**).

Lipophilicity assay

Lipophilicity is considered an important parameter for ABCB1 inhibitors. Therefore, an estimation of this parameter using both, experimental and *in silico* methods was conducted for the whole investigated series (**7-23**). Firstly, lipophilic properties were determined experimentally, followed by *in silico* prediction for the basic forms of compounds **7-23** with the use of four computer programs.

Lipophilicity estimation using RP-TLC method

Lipophilicity was determined experimentally using standard RP-TLC method. Results are presented as R_{M0} values (Figure 5) reflecting lipophilic properties on the basis of retention parameters, which are presented with details in Supporting Information (Table S1).

Moreover, changing from propyl (**18**) to ethyl (**17**) linkers increased R_{M0} value from 2.21 to 2.56. Interestingly, the highest results in both groups (2.99 and 3.35) were obtained for the 3-((4-fluoro)benzyloxy)benzylidene derivatives (**10** and **21**, respectively).

The lack of regular relationship between chemical structure and lipophilicity can be explained by extended polymorphism of compounds **7-23** proved by elemental analysis. The chemical characteristics (see Experimental, Chemical synthesis) indicate that these compounds form different crystallographic sequences, from mono- to trihydrochloric salts, simultaneously being hydrates (in content 0.5-3.5 of H_2O per molecule), excluding compound **16**. The interference of two opposing traits, *i.e.* the hydrophilicity-increasing hydrochloride form on one hand, and the hydrate-form strongly reducing hydrophilicity on the other, together with the lipophilicity

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resulting from a single molecule structure, seems to cause the irregular difference of the R_{M0} measured (Figure 5). The lowest lipophilicity observed for the only non-hydrate compound **16**, obtained in the triple-hydrochloride form, distinctly supports this theory. However, the resultant of the triple overlapping effects is not so easy to find for other members of the series (**7-15**, **17-23**).

Lipophilicity prediction in silico

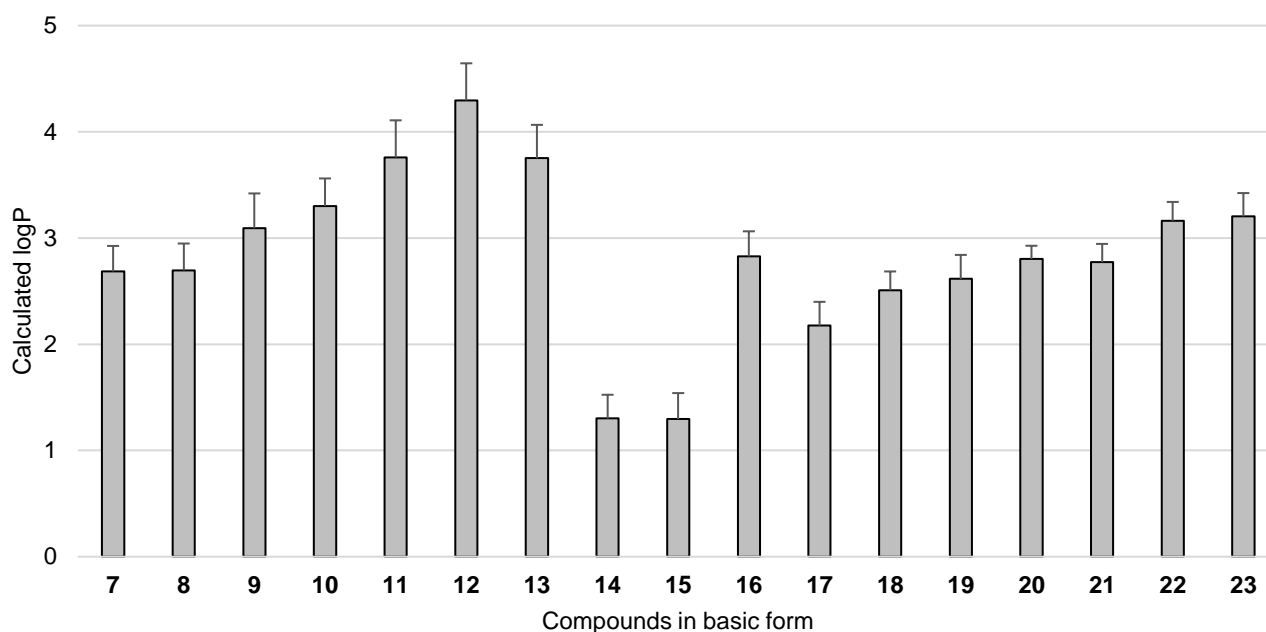


Figure 6. Results of lipophilicity from *in silico* prediction calculated for 5-arylideneimidazolones (**7-23**) in basic forms, presented as arithmetic mean from all obtained values with standard deviations.

The lowest mean values of logP were obtained by compounds with single aromatic ring at position 5 of imidazolone and only aliphatic moieties at other positions (**14** and **15**). For benzyloxybenzylidene derivatives from both groups A (**9-12**) and B (**17-23**), an increase of lipophilicity due to addition of halogen is clearly visible. Furthermore, compound **12** containing two chlorine atoms acquired the highest lipophilicity among all tested compounds. Compounds with amine only at position 2 demonstrated higher logP than those with amine at positions 2 and 3 (**9** vs **17**, **18**, and **19**; **10** vs **20**, **21**; **11** vs **22**, **23**; Group A vs B).

In contrast to experimental results for salts (**7-23**), results simulated for the basic form of this series clearly demonstrate the larger aromatic moiety (including hydrophobic substituents), the higher logP value of the whole molecule. Moreover, it can confirm the remark from the experimental test (Figure 5) that compounds with N-methylpiperazine have usually slightly higher logP than morpholine ones.

SAR discussion

The series of new potential ABCB1 modulators (**7-23**) was rationally designed on the basis of previously found active imidazolone derivatives [23,24]. The new compounds possess

In order to verify the role of polymorphism in matting the lipophilic properties of single molecule in the interaction with the ABCB1 pump at the molecular level, logP was calculated for the compounds (**7-23**) in the basic forms. Thus, *in silico* assays were performed with the use of four bioinformatics tools, *i.e.* SwissADME [32], pkCSM [33], molinspiration [34], and ALOGPS [35, 36]. Results, including mean value of logP and standard deviations from all used programs, are shown in Figure 6, while detailed data are presented in Supporting Information (Table S2).

various arylidene moieties at position 5, mostly 3-benzyloxybenzylidene fragments. They were divided into two groups, depending on the position of amine fragments, *i.e.* the amine fragment connected to C2 of imidazolone ring in case of group A (**7-13**, Table 1), and the amine group at position 2- and the alkylamine group connected to position N3 of the imidazolone ring in the case of group B (**14-23**, Table 2). Such diversity of fragments at position 2, 3 and 5 of imidazolone ring together with comprehensive biological results give an opportunity to perform structure-activity relationship studies. Thus, the role of the following structural traits can be considered: (i) a type of aromatic moiety at position 5, (ii) the amine position (group A vs group B) and (iii) a kind of amine (N-methylpiperazine vs morpholine vs phenylpiperazine) connected to N3 with the alkyl chain in group B.

In the cytotoxicity assays, three compounds (**14**, **15** and **23**) displayed strong effect in MDR T-lymphoma cancer cells (Table 3). All of them belong to the group B. The 4-fluorophenyl (**14**, **15**) seems to be the preferable aromatic moiety for the cytotoxic effect on MDR T-lymphoma cells, while the chlorobenzyloxybenzylidene compound **23** proved the nonselective cytotoxic action on both, MDR and PAR, cell lines. Referring to our previous research among imidazolones [24], this work is the first, in which we have taken into account either

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the single-ring aromatic moiety- or phenoxybenzylidene-containing compounds. An introduction of these new moieties indisputably improved the desirable anticancer profile of the series of arylidene-imidazolones giving selectivity for MDR cancer cell line with respect to the parental ones. This relevance was not observed earlier [24]. Hence, five tested compounds, *i.e.* the benzyloxybenzylidene derivatives **9**, **12**, **23**, and the fluorobenzylidene ones (**14**, **15**) could be considered selective anticancer agents (SI > 1.5; Table 3). The most selective compound **15** as well as compound **23** contain the N-methylpiperazine fragment, whereas the remained three ones (**9**, **12**, and **14**) are morpholine derivatives. Results for the fluorobenzylidene derivatives **14-16** allow to evaluate the role of the amine termination at position 3. Thus, non-aromatic amines (**14**, **15**), morpholine and N-methylpiperazine, seem to be preferable for the considered anticancer action comparing to the phenylpiperazine one (**16**). However, more fluorobenzylidene derivatives and more structural data are necessary in order to generalize this conclusion.

Results for compounds **7-23** tested in rhodamine 123 accumulation assay (Table 4) showed that the most active compounds (**12** and **22**) belonging to different groups (A and B, respectively), while one additional member of the group B (**20**) demonstrated moderate activity. Moreover, less active and inactive compounds can be found almost equally in both groups. Concerning amines in group B, the morpholine derivatives were usually more active ABCB1-modulators than N-methylpiperazine ones (**20** vs **21** and **22** vs **23**), and this observation is in a good agreement with our previous results [24]. However, for the 3-benzyloxybenzylidene moiety without substituents, the opposite result may be noticed. In the case of arylidene moieties, the final products with the 3-benzyloxybenzylidene substitution were more potent ABCB1-inhibitors than others investigated in this study, regardless of quantity of rings and the amine position. Furthermore, the addition of halogen to the phenyl ring seems to be beneficial. Interestingly, fluorine was preferable in the group A, while chlorine in group B, in the case of mono-substitution.

An insight into the ABCB1-modulating mechanism for the most active compounds (**12**, **20**, and **22**) proved their potent activity as ATPase substrates (Figure 3). This, in accordance with previous results [24], suggests a competitive action with the fluorescent substrate (123 rhodamine) as the most probable mechanism of the efflux inhibition caused by this series. However, the obtained ATPase-stimulation results for the 2,3-disubstituted compounds (**20**, **22**) were significantly higher than that of reference verapamil and most of the previous imidazolones tested [24].

The structure-activity relationship analysis for this series (**7-23**) gives three important conclusions concerning three compounds presented within this study. First, the benzyloxybenzylidene compound **12** (group A) seems to be particularly interesting due to the MDR-cancer selectivity (SI: 1.87) and the potent ABCB1 modulating properties (the significant FAR at 2 μ M, Table 4). Secondly, the fluorobenzylidene derivatives **14** and **15** have been found as very promising anticancer agents, even more potent than doxorubicin. Thirdly, their action was highly selective towards MDR cancer cell line (SI > 40) but, interestingly, both **14** and **15** did not influence ABCB1 efflux pump in the rhodamine 123 accumulation assay. Worth to underline, the second and third

conclusions are new attractive findings, which have not been noted in our previous studies [24].

On the other hand, the pharmacophore model elaborated for 5-arylideneimidazolones indicated five structural features (Figure 4A). Among them, two aromatic rings are the features in concordance with previously obtained results. Interestingly, the presence of chlorine in the most active compounds (**12**, **22**) does not fit in to any feature of the pharmacophore model. Probably, this feature was not included in the pharmacophore hypotheses due to the absence of halogens within lead structures (**4** and **5**, Figure 2) also used to define the pharmacophore model. However, the halogen at the phenyl moiety additionally increases the general size and lipophilic properties of the hydrophobic aromatic area, which may be beneficial for appropriate interactions with ABCB1 protein. Thus, it should be considered in further design, that halogen, mainly chlorine, could increase activity. This pharmacophore model also indicates three other features, *i.e.* hydrogen bond acceptor (HBA), hydrogen bond donor (HBD) and protonable nitrogen atom (PI). This points out an impact of the amine fragment spaced from imidazolone ring.

In the biological research performed, representatives of both groups, A and B, demonstrated the desirable activity. These groups did not show any regular relationship between lipophilic properties evaluated experimentally and their biological activities (Figure 5). It is only seen that group B demonstrated wider range of R_{M0} values. In general, compounds with N-methylpiperazine were more lipophilic than morpholine, if they contained two unfused rings at position 5 of the imidazolone ring. The opposite results, however, were observed for the fluorophenyl derivatives. In contrast to the previous studies concerning hydantoin derivatives [27], the resultant lipophilicity of compounds did not affect the ABCB1 modulating potency in this series (**7-23**). As the clearest example, the most lipophilic compound **21** did not demonstrate ABCB1-modulating action, while the most active modulator **22** showed in 1-unit lower lipophilicity than that of **21** (2.30 vs 3.35, Figure 5). However, the comparison of experimental lipophilic properties was not easy within this series due to the polymorphic variety, *i.e.* the different HCl salt-hydrate balance for particular compounds. Otherwise, the *in silico* simulation of lipophilic properties for the basic form of each member of the series (**7-23**) gave some support for these considerations. Thus, it can be seen that compounds with the lowest calculated logP (**14** and **15**) demonstrated the most potent cancer cytotoxicity in MDR cell line and significant selectivity index. However this deduction is not followed by other compounds, *e.g.* the compound **12** with SI > 1.5 acquired the highest calculated logP. In the case of relationship between logP and ABCB1 inhibiting properties, some pattern can be observed (Figure 6 vs Table 4). Compounds with the top FAR obtained either the highest (**12**, group A) or one of the highest (**22**, group B) results of the calculated logP. Compounds with the lowest logP (**7** and **8**, Group A; and **14**, **15**, **17**, and **18** from Group B) were also deprived of the ABCB1 modulating properties.

On the basis of the SAR analysis performed, the (i) 4-fluorophenyl moieties, which contain (ii) N-methylpiperazine or morpholine, placed in the topological mode of group B, occurred beneficial in search for selective anticancer agents inhibiting growth of the resistant cancer cells. Such compounds seem to be an interesting lead structures for further

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modifications in the search for new solutions to overcome cancer MDR. Furthermore, the compounds with (i) benzyloxybenzylidene substituent at position 5, which belong to (ii) either A or B topologic group and (iii) contain morpholine as the amine fragment, seem to be the most promising imidazolone-derived candidates for potential ABCB1-modulators.

Conclusion

In this work, the series of new 5-arylideneimidazolone derivatives (**7-23**) with amine at position 2 (Group A) or 2 and 3 (Group B), was explored in search for anticancer agents selectively active towards MDR cells and/or modulating ABCB1 efflux pump. These compounds were rationally designed and originally synthesized in the 3-5 steps synthetic pathways, followed by screening *in vitro* with the use of assays in PAR and MDR T-lymphoma cancer cells, involving MTT cytotoxicity- and rhodamine 123 accumulation assays, respectively. Active modulators found were also examined on their mode of action in Pgp-Glo™ Assay. Pharmacophore-based molecular modelling was performed as well as lipophilicity was examined using both experimental and *in silico* methods.

The obtained results pointed out two compounds as the most promising anticancer agents overcoming MDR, *i.e.* (Z)-2-amino-5-(4-fluorobenzylidene)-3-(3-morpholinopropyl)-4H-imidazol-4-one hydrochloride (**14**) and (Z)-2-amino-5-(4-fluorobenzylidene)-3-(3-(4-methylpiperazin-1-yl)propyl)-4H-imidazol-4-one hydrochloride (**15**). These compounds turned out to be selective towards MDR T-lymphoma, and more potent than doxorubicin anticancer agents in the MTT cytotoxicity assays. Furthermore, (Z)-5-(3-((2,4-dichlorobenzyl)oxy)benzylidene)-2-(4-morpholinopiperidin-1-yl)-3,5-dihydro-4H-imidazol-4-one hydrochloride (**12**) displayed the pump modulating activity nearly as potent as the third generation ABCB1 inhibitor, tariquidar, together with selective cytotoxic effects towards MDR cancer cells. Based on the biological results, new pharmacophore, postulating five structural features responsible for ABCB1 modulating properties in the considered chemical group, was designed. In view of the all results of this study, the considered chemical family of imidazolones seems to be very promising in search for new therapeutic approaches in the fight against resistant cancer diseases. Therefore, further extensive studies for the imidazolones are needed, in which compounds **12**, **14**, and **15** may serve as parallel lead structures.

Experimental Section

Chemical synthesis

Reagents were purchased from Sigma Aldrich (Darmstadt, Germany), Alfa Aesar (Karlsruhe, Germany) or Acros Organics (Geel, Belgium). Reactions progress were verified using thin layer chromatography (TLC). It was carried out on silica gel 60 F254 plates (0.2 mm Merck). UV light was used for spots visualization. MEL-TEMP II apparatus (LD Inc., Long Beach, CA, USA) was used to determine melting points (mp) and are uncorrected. The ¹H-NMR spectra were obtained on a Mercury-VX 300 Mz spectrometer (Varian, Palo Alto, CA, USA) or JOEL JNM-

ECZR500 RS1 (ECZR version) at 500 MHz. DMSO-d₆ was used as deuterated solvent and internal standard. Chemical shifts (δ) are given in ppm (parts per million). Data are reported in following order: chemical shift, multiplicity (s - singlet; br. s - broad singlet; d - doublet; d def. - doublet deformed; dd - doublet of doublets; t - triplet; t def. - triplet deformed; qui - quintet; m - multiplet), coupling constant J (Hz), protons' number, position of particular protons (Ar - aromatic moiety at C5 of imidazolone, Pip-Ar - aromatic moiety linked to piperazine, Phth - phthalimide, Pp - piperidine, Mor - morpholine, Pip - piperazine, Ar-Pip - piperazine linked with aromatic moiety). Mass spectra were obtained on a UPLC-MS/MS system consisted of a Waters ACQUITY@UPLC® (Waters Corporation, Milford, MA, USA) coupled to a Waters TQD mass spectrometer (electrospray ionization mode ESI-tandem quadrupole). Chromatographic separations were performed with Acquity UPLC BEH (bridged ethyl hybrid) C18 column; 2.1 × 100 mm, and 1.7 μm particle size, equipped with Acquity UPLC BEH C18 VanGuard precolumn (Waters Corporation, Milford, MA, USA), 2.1 × 5 mm and 1.7 μm particle size. The detailed separations conditions: temperature 40°C, elution under gradient from 95% to 0% of eluent A over 10 min, at a flow rate of 0.3 mL·min⁻¹. Eluent A: water/formic acid (0.1%, v/v); eluent B: acetonitrile/formic acid (0.1%, v/v). Waters eA PDA detector was used for chromatograms preparations. Spectra were analyzed in the range from 200 to 700 nm with 1.2 nm resolution and sampling rate 20 points/s. MS detection settings of Waters TQD mass spectrometer were as follows: source temperature 150°C, desolvation temperature 350°C, desolvation gas flow rate 600 L·h⁻¹, cone gas flow 100 L·h⁻¹, capillary potential 3.00 kV, cone potential 40 V. For nebulization and as drying gas, nitrogen was used. The appropriate data were obtained in a scan mode in range between 50 and 1000 m/z in time 0.5 s intervals. MassLynx V 4.1 (Waters Corporation, Milford, MA, USA) software was used for data acquisition. Retention times (t_R) are presented in minutes. The UPLC/MS analysis provided the purity information (%) of all the described compounds. Elemental analyses (C, H, N) for final compounds (**7-23**) were performed on an Elemental Analyser Vario El III (Hanau, Germany). Detailed procedure and synthesis conditions for intermediates **30**, **31**, **36**, **38**, **39**, **44**, and final product **7** were described previously [25,26]. Here, elemental analysis for compound **7** was additionally measured and the chemical formula estimated, as follows: anal. calcd for C₂₃H₂₆N₄O₂·3HCl·0.5H₂O: C, 54.28; H, 5.95; N, 11.01; found: C, 54.07; H, 6.10; N, 10.87.

General procedure to obtain aminepropylphthalimide derivative (**24**)

2-(3-Bromopropyl)isoindoline-1,3-dione (50 mmol, 13.40 g), 1-phenylpiperazine (50 mmol, 8.11 g), potassium carbonate (150 mmol, 20.73 g), TEBA (5 mmol, 1.15 g) and acetone (150 ml) were put into flat-bottom flask. The reaction mixture was heated under reflux and stirred for 5 hours. Then, it was stirred at room temperature for 72 hours. The reaction mixture was filtered off. The resulted filtrate was evaporated, extracted with methylene chloride, dried over magnesium sulphate. After that, filtration and evaporation were performed. Solid precipitated after cooling in the fridge.

2-(3-(4-Phenylpiperazin-1-yl)propyl)isoindoline-1,3-dione (**24**)

Yellow solid. Yield 66 %; mp 113-116 °C. C₂₁H₂₃N₃O₂ MW 349.43. ¹H-NMR (DMSO-d₆, ppm): δ 7.84 – 7.80 (m, 2H, Phth-4,5-H), 7.77 – 7.73 (m, 2H, Phth-6,7-H), 7.14 – 7.10 (m, 2H, Pip-Ar-3,5-H), 6.81 – 6.76 (m, 2H, Pip-Ar-2,6-H), 6.72 – 6.67 (m, 1H, Pip-Ar-4-H), 3.65-3.59 (t, J = 6.8 Hz, 2H, Pip-CH₂-CH₂-CH₂), 2.89-2.82 (t def., 4H, Ar-Pip-2,6-H), 2.38 – 2.29 (m, 6H, Ar-Pip-3,5-H, Pip-CH₂), 1.78-1.70 (qui, J = 6.7 Hz, 2H, Pip-CH₂-CH₂). LC/MSx: purity % t_R = 4.02, (ESI) m/z [M+H]⁺ 350.30.

4.1.2. General procedure to obtain primary amine from phthalimide derivative (**25**)

The mixture of 2-(3-(4-phenylpiperazin-1-yl)propyl)isoindoline-1,3-dione (**24**) (14.5 mmol, 5.07 g) and hydrazine (145 mmol, 7.26 g) in

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anhydrous ethanol (145 ml) was stirred and refluxed for 6 hours. Then, it was stirred at room temperature for 72 hours. After that, the reaction mixture was filtered off; the resulted filtrate was evaporated. Extraction with methylene chloride was performed. After drying over sodium sulphate, filtration and evaporation, pure product was obtained.

3-(4-Phenylpiperazin-1-yl)propan-1-amine (**25**)

Cream oil. Yield 53 %. $C_{13}H_{21}N_3$ MW 219.33. 1H -NMR (DMSO- d_6 , ppm): δ 7.21 – 7.11 (m, 2H, Pip-Ar-3,5-H), 6.86 (d def., 2H, Pip-Ar-2,6-H), 6.76 – 6.69 (m, 1H, Pip-Ar-4-H), 3.11-3.01 (t def., 4H, Ar-Pip-2,6-H, H_3O^+), 2.62 – 2.49 (t def., 2H, Pip- CH_2 - CH_2 - CH_2), 2.38-2.34 (t def., 4H, Ar-Pip-3,5-H), 2.34 – 2.20 (m, 2H, Pip- CH_2), 1.53-1.37 (m, 2H, Pip- CH_2 - CH_2). LC/MS \pm : purity 95 % t_R = 1.36, (ESI) m/z $[M+H]^+$ 220.24.

4.1.3. General procedure to obtain 3-benzyloxybenzaldehydes (**26-29**)

3-Hydroxybenzaldehyde (50 mmol, 6.11 g), benzyl chloride or benzyl bromide (50 mmol), potassium carbonate (25 mmol, 3.45 g) and ethanol (50 ml) was put into flask and refluxed for 6 hours. Then, the reaction mixture was cooled down to room temperature and filtered. The filtrate was evaporated and then extracted using methylene chloride and washed with 1 % NaOH (twice) and water (one time). It was dried over magnesium sulphate, filtered and evaporated.

3-(Benzyloxy)benzaldehyde (**26**)

3-Hydroxybenzaldehyde (50 mmol, 6.11 g) and benzyl chloride (50 mmol, 6.33 g) were used. White solid. Yield 36 %; mp 48-50 °C. $C_{14}H_{12}O_2$ MW 212.24. 1H -NMR (DMSO- d_6 , ppm): δ 9.97 (s, 1H, Ar-CHO), 7.56-7.51 (m, 3H, Ar-2,5,6-H), 7.49-7.45 (m, 2H, Ar'-2,6-H), 7.42-7.38 (m, 2H, Ar'-3,5-H), 7.37-7.32 (m, 2H, Ar-4-H, Ar'-4-H), 5.19 (s, 2H, O- CH_2). LC/MS \pm : purity 98.35 % t_R = 7.07, (ESI) m/z $[M+H]^+$ 213.20.

3-(4-Fluorobenzyloxy)benzaldehyde (**27**)

3-Hydroxybenzaldehyde (50 mmol, 6.11 g) and 4-fluorobenzyl chloride (50 mmol, 7.23 g) were used. Light orange solid. Yield 40 %; mp 58-60 °C. $C_{14}H_{11}FO_2$ MW 230.24. 1H -NMR (DMSO- d_6 , ppm): δ 9.94 (s, 1H, Ar-CHO), 7.88-7.39 (m, 5H, Ar-2,5,6-H, Ar'-2,6-H), 7.37-7.27 (m, 1H, Ar-4-H), 7.25-7.01 (t def., 2H, Ar'-3,5-H), 5.14 (s, 2H, O- CH_2). LC/MS \pm : purity 100.00 % t_R = 7.10, (ESI) m/z $[M+H]^+$ 231.14.

3-(4-Chlorobenzyloxy)benzaldehyde (**28**)

3-Hydroxybenzaldehyde (50 mmol, 6.11 g) and 4-chlorobenzyl bromide (50 mmol, 10.27 g) were used. White solid. Yield 19 %; mp 43-45 °C. $C_{14}H_{11}ClO_2$ MW 246.69. 1H -NMR (DMSO- d_6 , ppm): δ 9.94 (s, 1H, CHO), 7.51-7.48 (m, 2H, Ar'-3,5-H), 7.48-7.41 (m, 5H, Ar-2,4,6-H, Ar'-2,6-H), 7.34-7.29 (m, 1H, Ar-5-H), 5.16 (s, 2H, O- CH_2). LC/MS \pm : purity 96.69 % t_R = 7.76, (ESI) m/z $[M+H]^+$ 247.17.

3-(2,4-Dichlorobenzyloxy)benzaldehyde (**29**)

3-Hydroxybenzaldehyde (50 mmol, 6.11 g) and 2,4-dichlorobenzyl chloride (50 mmol, 9.77 g) were used. Cream solid. Yield 26 %; mp 69-71 °C. $C_{14}H_{10}Cl_2O_2$ MW 281.13. 1H -NMR (DMSO- d_6 , ppm): δ 9.95 (s, 1H, CHO), 7.76-7.67 (d def., 1H, Ar'-3-H), 7.65-7.60 (d def., 1H, Ar'-6-H), 7.57-7.52 (m, 2H, Ar-2,6-H), 7.50-7.49 (m, 1H, Ar-4-H), 7.47-7.40 (m, 1H, Ar'-5-H), 7.36-7.29 (m, 1H, Ar-5-H), 5.20 (s, 2H, O- CH_2). LC/MS \pm : purity 100.00 % t_R = 8.47, (ESI) m/z $[M+H]^+$ 281.26.

4.1.4. General procedure of Knoevenagel condensation (**32-35, 37**)

Thiohydantoin (10-31 mmol, 1.16-3.60 g), proper aldehyde (10-31 mmol), sodium acetate (10-31 mmol, 0.82-2.54 g), and acetic acid (10-31 ml) was added to flat-bottom flask and heated under reflux for 5.5 hours and then stirred at room temperature for next 20 hours. The reaction mixture was filtered to provide product which was further purified via crystallization from acetic acid if necessary.

(Z)-5-(3-(Benzyloxy)benzylidene)-2-thioxoimidazolidin-4-one (**32**)

2-Thiohydantoin (19 mmol, 2.20 g) and 3-(benzyloxy)benzaldehyde (**26**) (19 mmol, 4.03 g) were used. Brown solid. Yield 99 %; mp 211-213 °C. $C_{17}H_{14}N_2O_2S$ MW 310.37. 1H -NMR (DMSO- d_6 , ppm): δ 12.36 (s, 1H, N1-H), 12.17 (s, 1H, N3-H), 7.46-7.41 (m, 2H, Ar'-2,6-H), 7.39-7.35 (m, 2H, Ar'-3,5-H), 7.33-7.28 (m, 4H, Ar-2,5,6-H, Ar'-4-H), 7.02-6.97 (m, 1H, Ar-4-H), 6.41 (s, 1H, C=CH), 5.14 (s, 2H, O- CH_2). LC/MS \pm : purity 96.64 % t_R = 6.60, (ESI) m/z $[M+H]^+$ 311.22.

(Z)-5-(3-((4-Fluorobenzyloxy)benzylidene)-2-thioxoimidazolidin-4-one (**33**)

2-Thiohydantoin (20 mmol, 2.32 g) and 3-((4-fluorobenzyloxy)benzaldehyde (**27**) (20 mmol, 4.60 g) were used. Yellow solid. Yield 80 %; mp 220-223 °C. $C_{17}H_{13}FN_2O_2S$ MW 328.36. 1H -NMR (DMSO- d_6 , ppm): δ 12.27 (s, 1H, N1-H), 12.04 (s, 1H, N3-H), 7.73-7.67 (d def., 2H, Ar'-2,6-H), 7.51-7.44 (m, 2H, Ar-4,6-H), 7.23-7.15 (m, 2H, Ar-2,5-H), 7.04-6.98 (d def., 2H, Ar'-3,5-H), 6.43 (s, 1H, C=CH), 5.12 (s, 2H, O- CH_2). LC/MS \pm : purity 100.00 % t_R = 6.67, (ESI) m/z $[M+H]^+$ 329.17.

(Z)-5-(3-((4-Chlorobenzyloxy)benzylidene)-2-thioxoimidazolidin-4-one (**34**)

2-Thiohydantoin (10 mmol, 1.16 g) and 3-((4-chlorobenzyloxy)benzaldehyde (**28**) (10 mmol, 2.46 g) were used. Brown solid. Yield 79 %; mp 223-225 °C. $C_{17}H_{13}ClN_2O_2S$ MW 344.81. 1H -NMR (DMSO- d_6 , ppm): δ 12.36 (s, 1H, N1-H), 12.17 (s, 1H, N3-H), 7.48-7.40 (m, 4H, Ar'-2,3,5,6-H), 7.34-7.27 (m, 3H, Ar-2,5,6-H), 7.02-6.97 (m, 1H, Ar-4-H), 6.41 (s, 1H, C=CH), 5.14 (s, 2H, O- CH_2). LC/MS \pm : purity 98.70 % t_R = 7.23, (ESI) m/z $[M+H]^+$ 345.21.

(Z)-5-(3-((2,4-Dichlorobenzyloxy)benzylidene)-2-thioxoimidazolidin-4-one (**35**)

2-Thiohydantoin (31 mmol, 3.60 g) and 3-((2,4-dichlorobenzyloxy)benzaldehyde (**29**) (31 mmol, 8.72 g) were used. Yellow solid. Yield 88 %; mp 190-192 °C. $C_{17}H_{12}Cl_2N_2O_2S$ MW 379.26. 1H -NMR (DMSO- d_6 , ppm): δ 12.37 (s, 1H, N1-H), 12.20 (s, 1H, N3-H), 7.88-7.67 (d def., 1H, Ar'-3-H), 7.62-7.58 (d def., 1H, Ar'-6-H), 7.54-7.45 (m, 1H, Ar'-5-H), 7.41-7.11 (m, 3H, Ar-2,5,6-H), 7.06-6.84 (m, 1H, Ar-4-H), 6.42 (s, 1H, C=CH), 5.18 (s, 2H, O- CH_2). LC/MS \pm : purity 99.39 % t_R = 7.92, (ESI) m/z $[M+H]^+$ 379.09.

(Z)-5-(4-Fluorobenzyloxy)-2-thioxoimidazolidin-4-one (**37**)

2-Thiohydantoin (25 mmol, 2.90 g) and 4-fluorobenzaldehyde (25 mmol, 3.10 g) were used. Yellow solid. Yield 84 %; mp 253-257 °C. $C_{10}H_7FN_2OS$ MW 222.24. 1H -NMR (DMSO- d_6 , ppm): δ 12.64-11.98 (d def., 2H, N1-H, N3-H), 7.87-7.68 (m, 2H, Ar-2,6-H), 7.32-7.14 (m, 2H, Ar-3,5-H), 6.46 (s, 1H, C=CH). LC/MS \pm : purity 100.00 % t_R = 4.47, (ESI) m/z $[M+H]^+$ 223.04.

4.1.5. General procedure of S-methylation (**40-43, 45**)

Firstly, sodium (0.16-0.61 g, 7-26.5 mmol) was dissolved in ethanol (10.85-39.75 mL). To this solution, appropriate 5-arylidenthiohydantoin (7-26.5 mmol) was added. After 3 minutes, iodomethane (1.06-4.03 g, 7-26.5 mmol) was added. The reaction

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mixture was stirred at room temperature for 24 hours. Filtration was performed to provide product, which was further purified via crystallization from acetone, if necessary.

(Z)-5-(3-(Benzyloxy)benzylidene)-2-(methylthio)-3,5-dihydro-4H-imidazol-4-one (**40**)

(Z)-5-(3-(Benzyloxy)benzylidene)-2-thioxoimidazolidin-4-one (**32**) (17 mmol, 5.28 g) and iodomethane (17 mmol, 2.58 g) were used. Yellow solid. Yield 48 %; mp 233-235 °C. $C_{18}H_{16}N_2O_2S$ MW 324.40. 1H -NMR (DMSO- d_6 , ppm): δ 11.81 (s, 1H, N3-H), 8.00 (s, 1H, Ar-2-H), 7.67-7.62 (d def., 1H, Ar-6-H), 7.44-7.41 (m, 2H, Ar'-2,6-H), 7.39-7.34 (m, 2H, Ar'-3,5-H), 7.33-7.27 (m, 2H, Ar-5-H, Ar'-4-H), 7.02-6.97 (m, 1H, Ar-4-H), 6.67 (s, 1H, C=CH), 5.11 (s, 2H, O-CH₂), 2.61 (s, 3H, CH₃). LC/MS \pm : purity 97.52 % t_R = 7.39, (ESI) m/z [M+H]⁺ 325.26.

(Z)-5-(3-((4-fluorobenzyl)oxy)benzylidene)-2-(methylthio)-3,5-dihydro-4H-imidazol-4-one (**41**)

(Z)-5-(3-((4-Fluorobenzyl)oxy)benzylidene)-2-thioxoimidazolidin-4-one (**33**) (16 mmol, 5.25 g) and iodomethane (16 mmol, 2.43 g) were used. Yellow solid. Yield 30 %; mp 180-182 °C. $C_{18}H_{15}FN_2O_2S$ MW 342.39. 1H -NMR (DMSO- d_6 , ppm): δ 11.82 (s, 1H, N3-H), 7.99 (s, 1H, Ar-2-H), 7.68-7.64 (d, J = 13.8 Hz, 1H, Ar-6-H), 7.50 – 7.45 (m, 2H, Ar'-2,6-H), 7.33-7.28 (t, J = 22.4 Hz, 1H, Ar-5-H), 7.21 – 7.16 (m, 2H, Ar'-3,5-H), 6.98 (m, 1H, Ar-4-H), 6.67 (s, 1H, C=CH), 5.09 (s, 2H, O-CH₂), 2.61 (s, 3H, CH₃). LC/MS \pm : purity 94.32 % t_R = 7.40 (ESI) m/z [M+H]⁺ 343.19.

(Z)-5-(3-((4-Chlorobenzyl)oxy)benzylidene)-2-(methylthio)-3,5-dihydro-4H-imidazol-4-one (**42**)

(Z)-5-(3-((4-Chlorobenzyl)oxy)benzylidene)-2-thioxoimidazolidin-4-one (**34**) (7 mmol, 2.41 g) and iodomethane (7 mmol, 1.06 g) were used. Yellow solid. Yield 83 %; mp 108-110 °C. $C_{18}H_{15}ClN_2O_2S$ MW 358.84. 1H -NMR (DMSO- d_6 , ppm): δ 11.83 (s, 1H, N3-H), 7.98 (s, 1H, Ar-2-H), 7.87-7.62 (d def., 1H, Ar-6-H), 7.61-7.34 (m, 4H, Ar'-2,3,5,6-H), 7.32-7.14 (t def., 1H, Ar-5-H), 7.05-6.89 (m, 1H, Ar-4-H), 6.66 (s, 1H, C=CH), 5.11 (s, 2H, O-CH₂), 2.60 (s, 3H, CH₃). LC/MS \pm : purity 95.17 % t_R = 8.00, (ESI) m/z [M+H]⁺ 359.19.

(Z)-5-(3-((2,4-Dichlorobenzyl)oxy)benzylidene)-2-(methylthio)-3,5-dihydro-4H-imidazol-4-one (**43**)

(Z)-5-(3-((2,4-Dichlorobenzyl)oxy)benzylidene)-2-thioxoimidazolidin-4-one (**35**) (26.5 mmol, 10.05 g) and iodomethane (26.5 mmol, 4.03 g) were used. Yellow solid. Yield 61 %; mp 181-183 °C. $C_{18}H_{14}Cl_2N_2O_2S$ MW 393.28. 1H -NMR (DMSO- d_6 , ppm): δ 8.08 (s, 1H, Ar-2-H), 7.68-7.64 (d, J = 2.1 Hz, 1H, Ar'-3-H), 7.61-7.58 (d, J = 8.3 Hz, Ar'-6-H), 7.57-7.53 (d, J = 7.7 Hz, Ar'-5-H), 7.48-7.43 (dd, J = 8.3, 2.3 Hz, 1H, Ar-6-H), 7.31-7.25 (t, J = 7.9 Hz, 1H, Ar-5-H), 6.97-6.92 (dd, J = 8.0, 2.3 Hz, 1H, Ar-4-H), 6.48 (s, 1H, C=CH), 5.14 (s, 2H, O-CH₂), 2.54 (s, 3H, CH₃). LC/MS \pm : purity 92.74 % t_R = 8.88 (ESI) m/z [M+H]⁺ 393.10.

(Z)-5-(4-Fluorobenzylidene)-2-(methylthio)-4H-imidazol-4-one (**45**)

(Z)-5-(4-Fluorobenzylidene)-2-thioxoimidazolidin-4-one (**37**) (20 mmol, 4.44 g) and iodomethane (20 mmol, 3.04 g) were used. Yellow solid. Yield 97 %; mp 242-245 °C. $C_{11}H_9FN_2OS$ MW 236.26. 1H -NMR (DMSO- d_6 , ppm): δ 11.80 (br. s, 1H, N3-H), 8.32-8.14 (m, 2H, Ar-2,6-H), 7.32-7.15 (m, 2H, Ar-3,5-H), 6.72 (s, 1H, C=CH), 2.62 (s, 3H, CH₃). LC/MS \pm : purity 98.36 % t_R = 5.92, (ESI) m/z [M+H]⁺ 237.12.

4.1.6. General procedure to obtain 5-arylideneimidazolone final products (**8-23**)

(Z)-2-(Methylthio)-5-arylidene-4H-imidazol-4-one (2-5 mmol) and proper amine (2.5-6 mmol) were heated and stirred in oil bath with

controlled temperature (125 °C) for 15 minutes. After that time, ethanol (15 ml) was added. The mixture was stirred and heated under reflux for 5-6 hours. Then, it was stirred at room temperature for 20 hours. After that, gaseous hydrochloride acid was used for conversion of obtained products into hydrochloride form. If necessary, purification was performed using crystallization from ethanol.

(Z)-5-(Naphthalen-2-ylmethylene)-2-(4-morpholinopiperidin-1-yl)-3,5-dihydro-4H-imidazol-4-one hydrochloride (**8**)

(Z)-5-(Naphthalen-2-ylmethylene)-2-(methylthio)-3,5-dihydro-4H-imidazol-4-one (**39**) (5 mmol, 1.34 g) and 4-morpholinopiperidine (6 mmol, 1.02 g) were used. Yellow solid. Yield 98 %; mp 215-217 °C. $C_{23}H_{26}N_4O_2 \cdot 2HCl \cdot 1.5H_2O$ MW 490.42. 1H -NMR (DMSO- d_6 , ppm): δ 11.82 (s, 1H, NH⁺), 8.38 (s, 1H, N3-H), 8.11-7.98 (d def., 1H, Ar-1-H), 7.96-7.82 (m, 4H, Ar-3,4,5,8-H), 7.57-7.46 (m, 2H, Ar-6,7-H), 6.77 (s, 1H, C=CH), 4.90-3.61 (m, 6H, Mor-2,6-H_b, Mor-3,5-H, H₃O⁺), 3.55-3.43 (m, 1H, Pp-4-H), 3.41-3.33 (d def., 2H, Mor-2,6-H_a), 3.29-3.16 (t, J = 12.3 Hz, 2H, Pp-2,6-H_b), 3.13-2.97 (m, 2H, Pp-2,6-H_a), 2.33-2.22 (d def., 2H, Pp-3,5-H_b), 1.91-1.77 (m, 2H, Pp-3,5-H_a). LC/MS \pm : purity 100.00 % t_R = 3.97, (ESI) m/z [M+H]⁺ 391.35. Anal. calcd for $C_{23}H_{26}N_4O_2 \cdot 2HCl \cdot 1.5H_2O$: C, 56.33; H, 6.38; N, 11.43; found: C, 56.02; H, 6.29; N, 11.29.

(Z)-5-(3-(Benzyloxy)benzylidene)-2-(4-morpholinopiperidin-1-yl)-3,5-dihydro-4H-imidazol-4-one hydrochloride (**9**)

(Z)-5-(3-(Benzyloxy)benzylidene)-2-(methylthio)-3,5-dihydro-4H-imidazol-4-one (**40**) (5 mmol, 1.62 g) and 4-morpholinopiperidine (6 mmol, 1.02 g) were used. Yellow solid. Yield 91 %; mp 230-232 °C. $C_{26}H_{30}N_4O_3 \cdot 2HCl \cdot 1.5H_2O$ MW 546.49. 1H -NMR (DMSO- d_6 , ppm): δ 11.85 (s, 1H, NH⁺), 7.55 (s, 1H, N3-H), 7.46-7.34 (m, 6H, Ar-2,6-H, Ar'-2,3,5,6-H), 7.32-7.27 (m, 2H, Ar-5-H, Ar'-4-H), 7.00-6.94 (dd, J = 8.2, 2.1 Hz, 1H, Ar-4-H), 6.57 (s, 1H, C=CH), 5.13 (s, 2H, O-CH₂), 3.94-3.85 (m, 6H, Mor-3,5-H, Mor-2,6-H_b, H₃O⁺), 3.51-3.41 (m, 1H, Pp-4-H), 3.39-3.29 (d def., 2H, Mor-2,6-H_a), 3.24-3.11 (t, J = 12.5 Hz, 2H, Pp-2,6-H_b), 3.08-2.93 (m, 2H, Pp-2,6-H_a), 2.32-2.18 (d def., 2H, Pp-3,5-H_b), 1.91-1.73 (m, 2H, Pp-3,5-H_a). LC/MS \pm : purity 97.19 % t_R = 4.75, (ESI) m/z [M+H]⁺ 447.40. Anal. calcd for $C_{26}H_{30}N_4O_3 \cdot 2HCl \cdot 1.5H_2O$: C, 57.14; H, 6.47; N, 10.25; found: C, 57.11; H, 6.33; N, 10.21.

(Z)-5-(3-(4-Fluorobenzylidene)-2-(4-morpholinopiperidin-1-yl)-3,5-dihydro-4H-imidazol-4-one hydrochloride (**10**)

(Z)-5-(3-(4-Fluorobenzylidene)-2-(methylthio)-3,5-dihydro-4H-imidazol-4-one (**41**) (3 mmol, 1.03 g) and 4-morpholinopiperidine (3.5 mmol, 0.60 g) were used. Yellow solid. Yield 86 %; mp 228-230 °C. $C_{26}H_{29}FN_4O_3 \cdot 2HCl \cdot H_2O$ MW 555.47. 1H -NMR (DMSO- d_6 , ppm): δ 11.76 (s, 1H, NH⁺), 7.87-7.76 (d, J = 7.8 Hz, 2H, Ar'-2,6-H), 7.53-7.44 (m, 2H, Ar-2,6-H), 7.24-7.15 (m, 2H, Ar-4,5-H), 7.07-7.00 (d, J = 7.0 Hz, 2H, Ar'-3,5-H), 6.63 (s, 1H, C=CH), 5.12 (s, 2H, O-CH₂), 4.51-2.89 (m, 13H, Pp-2,4,6-H, Mor, H₃O⁺), 2.29-2.20 (d def., 2H, Pp-3,5-H_b), 1.89-1.73 (m, 2H, Pp-3,5-H_a). LC/MS \pm : purity 98.57 % t_R = 4.55, (ESI) m/z [M+H]⁺ 465.35. Anal. calcd for $C_{26}H_{29}FN_4O_3 \cdot 2HCl \cdot H_2O$: C, 56.22; H, 6.00; N, 10.09; found: C, 55.85; H, 5.76; N, 9.88.

(Z)-5-(3-(4-Chlorobenzylidene)-2-(4-morpholinopiperidin-1-yl)-3,5-dihydro-4H-imidazol-4-one hydrochloride (**11**)

(Z)-5-(3-(4-Chlorobenzylidene)-2-(methylthio)-3,5-dihydro-4H-imidazol-4-one (**42**) (3 mmol, 1.08 g) and 4-morpholinopiperidine (4 mmol, 0.68 g) were used. Cream solid. Yield 88 %; mp 208-210 °C. $C_{26}H_{29}ClN_4O_3 \cdot 2HCl \cdot 2.5H_2O$ MW 598.94. 1H -NMR (DMSO- d_6 , ppm): δ 11.76 (s, 1H, NH⁺), 7.56 (s, 1H, N3-H), 7.47-7.39 (m, 6H, Ar-2,6-H, Ar'-2,3,5,6-H), 7.32-7.27 (t, J = 8.0 Hz, 1H, Ar-5-H), 6.97-6.92 (dd, J = 8.2, 2.0 Hz, 1H, Ar-4-H), 6.53 (s, 1H, C=CH), 5.13 (s, 2H, O-CH₂), 3.96-3.83 (m, 6H, Mor-3,5-H, Mor-2,6-H_b, H₃O⁺), 3.51-3.41 (m, 1H, Pp-4-H), 3.39-

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3.32 (d def., 2H, Mor-2,6-H_a), 3.21-3.10 (t, *J* = 11.9 Hz, 2H, Pp-2,6-H_b), 3.08-2.99 (m, 2H, Pp-2,6-H_a), 2.29-2.20 (d def., 2H, Pp-3,5-H_b), 1.84-1.73 (m, 2H, Pp-3,5-H_a). LC/MS±: purity 100.00 % *t_R* = 5.19, (ESI) *m/z* [M+H]⁺ 481.50. Anal. calcd for C₂₆H₂₉ClN₄O₃·2HCl·2.5H₂O: C, 52.14; H, 6.07; N, 9.36; found: C, 52.06; H, 5.89; N, 9.26.

(Z)-5-(3-(2,4-Dichlorobenzoyloxy)benzylidene)-2-(4-morpholinopiperidin-1-yl)-3,5-dihydro-4H-imidazol-4-one hydrochloride (**12**)

(Z)-5-(3-(2,4-Dichlorobenzoyloxy)benzylidene)-2-(methylthio)-3,5-dihydro-4H-imidazol-4-one (**43**) (3 mmol, 1.18 g) and 4-morpholinopiperidine (3.5 mmol, 0.60 g) were used. Cream solid. Yield 56 %; mp 213-215 °C. C₂₆H₂₈Cl₂N₄O₃·2HCl·3.5H₂O MW 651.41. ¹H-NMR (DMSO-*d*₆, ppm): δ 11.71 (s, 1H, NH⁺), 7.70-7.67 (d, *J* = 2.1 Hz, 1H, Ar-2-H), 7.67-7.62 (m, 1H, Ar'-3-H), 7.59-7.55 (d, *J* = 8.3 Hz, 1H, Ar'-5-H), 7.48-7.44 (dd, *J* = 8.3, 2.1 Hz, Ar-6-H), 7.42-7.38 (d def. 1H, Ar'-6-H), 7.35-7.28 (t, *J* = 7.9 Hz, 1H, Ar-5-H), 7.01-6.95 (dd, *J* = 8.0, 1.8 Hz, 1H, Ar-4-H), 6.53 (s, 1H, C=CH), 5.18 (s, 2H, O-CH₂), 3.99-3.82 (m, 6H, Mor-3,5-H, Mor-2,6-H_b, H₃O⁺), 3.50-3.41 (m, 1H, Pp-4-H), 3.39-3.30 (d def., 2H, Mor-2,6-H_a), 3.22-2.99 (m, 4H, Pp-2,6-H), 2.28-2.19 (d def., 2H, Pp-3,5-H_b), 1.85-1.73 (m, 2H, Pp-3,5-H_a). LC/MS±: purity 94.19 % *t_R* = 5.70, (ESI) *m/z* [M+H]⁺ 515.33. Anal. calcd for C₂₆H₂₈Cl₂N₄O₃·2HCl·3.5H₂O: C, 47.94; H, 5.74; N, 8.60; found: C, 47.96; H, 5.33; N, 8.42.

(Z)-5-(Phenanthren-9-ylmethylene)-2-(4-morpholinopiperidin-1-yl)-3,5-dihydro-4H-imidazol-4-one hydrochloride (**13**)

(Z)-5-(Phenanthren-9-ylmethylene)-2-(methylthio)-3,5-dihydro-4H-imidazol-4-one (**44**) (5 mmol, 1.60 g) and 4-morpholinopiperidine (6 mmol, 1.02 g) were used. Yellow solid. Yield 92 %; mp 275-277 °C. C₂₇H₂₈N₄O₂·3HCl·3.5H₂O MW 612.98. ¹H-NMR (DMSO-*d*₆, ppm): δ 11.89 (s, 1H, NH⁺), 8.93-8.86 (d def., 1H, Ar-1-H), 8.85-8.80 (d, *J* = 8.8 Hz, 1H, Ar-8-H), 8.43 (s, 1H, N3-H), 8.17-8.11 (d def., 1H, Ar-4-H), 8.03-7.96 (d def., 1H, Ar-5-H), 7.77-7.61 (m, 5H, Ar-2,3,6,7,10-H), 7.29 (s, 1H, C=CH), 4.66-2.98 (m, 13H, Pp-2,4,6-H, Mor, H₃O⁺), 2.32-2.20 (d def., 2H, Pp-3,5-H_b), 1.98-1.74 (m, 2H, Pp-3,5-H_a). LC/MS±: purity 97.37 % *t_R* = 4.51, (ESI) *m/z* [M+H]⁺ 441.35. Anal. calcd for C₂₇H₂₈N₄O₂·3HCl·3.5H₂O: C, 52.90; H, 6.26; N, 9.14; found: C, 52.97; H, 5.75; N, 9.03.

(Z)-2-Amino-5-(4-fluorobenzylidene)-3-(3-morpholinopropyl)-4H-imidazol-4-one hydrochloride (**14**)

(Z)-5-(4-Fluorobenzylidene)-2-(methylthio)-4H-imidazol-4-one (**45**) (5 mmol, 1.18 g) and 3-morpholinopropan-1-amine (6 mmol, 0.87 g) were used. Yellow solid. Yield 60 %; mp 248-251 °C. C₁₇H₂₁FN₄O₂·2HCl·H₂O MW 423.31 ¹H-NMR (DMSO-*d*₆, ppm): δ 8.05 (s, 2H, Ar-2,6-H), 7.42 (br. s, 1H, N3-H), 7.19-7.09 (t, *J* = 8.8 Hz, 2H, Ar-3,5-H), 6.23 (s, 1H, C=CH), 3.58-3.52 (t, *J* = 4.6 Hz, 4H, Mor-3,5-H), 3.42-3.11 (m, 4H, Mor-2,6-H, H₃O⁺), 2.39-2.14 (m, 4H, N3-CH₂-CH₂-CH₂), 1.68 (s, 2H, N3-CH₂-CH₂). LC/MS±: purity 98.64 % *t_R* = 2.93, (ESI) *m/z* [M+H]⁺ 333.29. Anal. calcd for C₁₇H₂₁FN₄O₂·2HCl·H₂O: C, 48.23; H, 5.96; N, 13.24; found: C, 48.52; H, 5.75; N, 13.34.

(Z)-2-Amino-5-(4-fluorobenzylidene)-3-(3-(4-methylpiperazin-1-yl)propyl)-4H-imidazol-4-one hydrochloride (**15**)

(Z)-5-(4-Fluorobenzylidene)-2-(methylthio)-4H-imidazol-4-one (**45**) (5 mmol, 1.18 g) and 3-(4-methylpiperazin-1-yl)propan-1-amine (6 mmol, 0.94 g) were used. Yellow solid. Yield 99 %; mp 254-257 °C. C₁₈H₂₄FN₅O·3HCl·3H₂O MW 508.84. ¹H-NMR (DMSO-*d*₆, ppm): δ 8.04 (s, 2H, Ar-2,6-H), 7.41 (br. s, 1H, N3-H), 7.21-7.08 (t def., 2H, Ar-3,5-H), 6.22 (s, 1H, C=CH), 3.32 (s, 2H, N3-CH₂, H₃O⁺), 2.42-2.15 (m, 10H, Pip, N3-CH₂-CH₂-CH₂), 2.12 (s, 3H, Pip-N-CH₃), 1.67 (s, 2H, N3-CH₂-CH₂). LC/MS±: purity 98.50 % *t_R* = 2.56, (ESI) *m/z* [M+H]⁺ 346.31. Anal.

calcd for C₁₈H₂₄FN₅O·3HCl·3H₂O: C, 42.48; H, 6.55; N, 13.77; found: C, 42.73; H, 6.05; N, 14.06.

(Z)-2-Amino-5-(4-fluorobenzylidene)-3-(3-(4-phenylpiperazin-1-yl)propyl)-3,5-dihydro-4H-imidazol-4-one hydrochloride (**16**)

(Z)-5-(4-Fluorobenzylidene)-2-(methylthio)-4H-imidazol-4-one (**45**) (3 mmol, 0.71 g) and 3-(4-phenylpiperazin-1-yl)propan-1-amine (**25**) (4 mmol, 0.88 g) were used. Yellow solid. Yield 81 %; mp 188-190 °C. C₂₃H₂₆FN₅O·3HCl MW 516.87. ¹H-NMR (DMSO-*d*₆, ppm): δ 11.35 (br. s, 1H, NH⁺), 9.56 (s, 1H, N3-H), 7.91-7.75 (m, 2H, Ar-2,6-H), 7.33-7.19 (m, 4H, Ar-3,5-H, Pip-Ar-3,5-H), 7.00-6.95 (d def., 2H, Pip-Ar-2,6-H), 6.85-6.76 (m, 2H, C=CH, Pip-Ar-4-H), 3.87-3.42 (m, 6H, N-CH₂, Ar-Pip-2,6-H), 3.29-2.99 (m, 6H, N-CH₂-CH₂-CH₂, Ar-Pip-3,5-H), 2.20-2.01 (m, 2H, N-CH₂-CH₂). LC/MS±: purity 99.74 % *t_R* = 4.50, (ESI) *m/z* [M+H]⁺ 408.39. Anal. calcd for C₂₃H₂₆FN₅O·3HCl: C, 53.44; H, 5.67; N, 13.55; found: C, 53.35; H, 5.69; N, 13.51.

(Z)-2-Amino-5-(3-(benzyloxy)benzylidene)-3-(2-morpholinoethyl)-3,5-dihydro-4H-imidazol-4-one hydrochloride (**17**)

(Z)-5-(3-(Benzyloxy)benzylidene)-2-(methylthio)-3,5-dihydro-4H-imidazol-4-one (**40**) (4 mmol, 1.30 g) and 2-morpholinoethan-1-amine (6 mmol, 0.79 g) were used. Cream solid. Yield 80 %; mp 203-205 °C. C₂₃H₂₆N₄O₃·2HCl·H₂O MW 497.42. ¹H-NMR (DMSO-*d*₆, ppm): δ 11.43 (br. s, 1H, NH⁺), 8.42 (br. s, 1H, N3-H), 7.55-7.41 (m, 3H, Ar-2-H, Ar'-2,6-H), 7.39-7.24 (m, 5H, Ar-5,6-H, Ar'-3,4,5-H), 7.09-6.86 (m, 1H, Ar-4-H), 6.71 (br. s, 1H, C=CH), 5.21-5.09 (m, 2H, O-CH₂), 4.00-3.66 (d def., 6H, N-CH₂, Mor-3,5-H) 3.50-2.96 (m, 6H, N3-CH₂-CH₂, Mor-2,6-H, H₃O⁺). LC/MS±: purity 98.62 % *t_R* = 4.70, (ESI) *m/z* [M+H]⁺ 407.50. Anal. calcd for C₂₃H₂₆N₄O₃·2HCl·H₂O: C, 55.53; H, 6.09; N, 11.27; found: C, 55.50; H, 6.01; N, 11.68.

(Z)-2-Amino-5-(3-(benzyloxy)benzylidene)-3-(3-morpholinopropyl)-3,5-dihydro-4H-imidazol-4-one hydrochloride (**18**)

(Z)-5-(3-(Benzyloxy)benzylidene)-2-(methylthio)-3,5-dihydro-4H-imidazol-4-one (**40**) (3 mmol, 0.97 g) and 3-morpholinopropan-1-amine (3.5 mmol, 0.50 g) were used. Yellow solid. Yield 77 %; mp 150-152 °C. C₂₄H₂₈N₄O₃·2HCl·1.5H₂O MW 520.45. ¹H-NMR (DMSO-*d*₆, ppm): δ 11.28 (br. s, 1H, NH⁺), 9.51 (br. s, 1H, N3-H), 7.48-7.40 (d def., 2H, Ar'-2,6-H), 7.39-7.23 (m, 6H, Ar-2,5,6-H, Ar'-3,4,5-H), 7.07-6.93 (m, 1H, Ar-4-H), 6.70 (s, 1H, C=CH), 5.23-5.08 (m, 2H, O-CH₂), 3.99-2.80 (m, 12H, Mor, N3-CH₂, N3-CH₂-CH₂-CH₂, H₃O⁺), 2.11-1.96 (m, 2H, N3-CH₂-CH₂). LC/MS±: purity 97.01 % *t_R* = 4.74, (ESI) *m/z* [M+H]⁺ 421.53. Anal. calcd for C₂₄H₂₈N₄O₃·2HCl·1.5H₂O: C, 55.38; H, 6.40; N, 10.77; found: C, 54.95; H, 6.24; N, 10.69.

(Z)-2-Amino-5-(3-(benzyloxy)benzylidene)-3-(3-(4-methylpiperazin-1-yl)propyl)-3,5-dihydro-4H-imidazol-4-one hydrochloride (**19**)

(Z)-5-(3-(Benzyloxy)benzylidene)-2-(methylthio)-3,5-dihydro-4H-imidazol-4-one (**40**) (4 mmol, 1.30 g) and 3-(4-methylpiperazin-1-yl)propan-1-amine (5.5 mmol, 0.865 g) were used. Cream solid. Yield 41 %; mp 229-231 °C. C₂₅H₃₁N₅O₃·3HCl·0.5H₂O MW 551.94. ¹H-NMR (DMSO-*d*₆, ppm): δ 12.14 (br. s, 1H, NH⁺), 9.66 (s, 1H, N3-H), 7.64-7.23 (m, 8H, Ar-2,5,6-H, Ar'-2,3,4,5,6-H), 7.09-6.94 (d def., 1H, Ar-4-H), 6.78-6.61 (d def., 1H, C=CH), 5.27-5.10 (d def., 2H, O-CH₂), 4.39-2.99 (m, 12H, Pip, N3-CH₂, N3-CH₂-CH₂-CH₂, 2.79 (s, 3H, Pip-N-CH₃), 2.03 (s, 2H, N3-CH₂-CH₂), 12.15 (br. s, 1H, NH⁺), 9.66 (s, 1H, NH), 7.60-7.24 (m, 9H, Ar-H, Ar'-H), 7.09-6.59 (d def., 1H, C=NH), 6.78-6.73 (d def., 1H, C=CH), 5.28-5.09 (d def., 2H, O-CH₂), 3.98-3.04 (m, 12H, Pip, N3-CH₂, N3-CH₂-CH₂-CH₂, H₃O⁺), 2.79 (s, 3H, Pip-N-CH₃), 2.03 (s, 2H, N3-CH₂-CH₂). LC/MS±: purity 98.73 % *t_R* = 4.18, (ESI) *m/z* [M+H]⁺ 434.38. Anal. calcd for C₂₅H₃₁N₅O₃·3HCl·0.5H₂O: C, 54.40; H, 6.40; N, 12.69; found: C, 54.74; H, 6.27; N, 12.64.

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(Z)-2-Amino-5-(3-(4-fluorobenzoyloxy)benzylidene)-3-(3-morpholinopropyl)-3,5-dihydro-4H-imidazol-4-one hydrochloride (**20**)

(Z)-5-(3-(4-Fluorobenzoyloxy)benzylidene)-2-(methylthio)-3,5-dihydro-4H-imidazol-4-one (**41**) (3 mmol, 1.03 g) and 3-morpholinopropan-1-amine (4 mmol, 0.58 g) were used. Yellow solid. Yield 57 %; mp 201-203 °C. $C_{24}H_{27}FN_4O_3 \cdot 2HCl \cdot H_2O$ MW 529.43. 1H -NMR (DMSO- d_6 , ppm): δ 9.31 (s, 1H, N3-H), 7.70 (s, 2H, Ar-2,5-H), 7.51-7.44 (m, 2H, Ar'-2,6-H), 7.22-7.16 (m, 2H, Ar'-3,5-H), 7.12-7.01 (d def., 2H, Ar-4,6-H), 6.73 (s, 1H, C=CH), 5.13 (s, 2H, O-CH₂), 4.01-2.82 (m, 12H, Mor, N3-CH₂, N3-CH₂-CH₂-CH₂, H₃O⁺), 2.02 (s, 2H, N3-CH₂-CH₂). LC/MS±: purity 98.65 % $t_R = 4.53$, (ESI) m/z $[M+H]^+$ 439.36. Anal. calcd for $C_{24}H_{27}FN_4O_3 \cdot 2HCl \cdot H_2O$: C, 54.44; H, 5.91; N, 10.58; found: C, 54.63; H, 5.80; N, 10.56.

(Z)-2-Amino-5-(3-(4-fluorobenzoyloxy)benzylidene)-3-(3-(4-methylpiperazin-1-yl)propyl)-3,5-dihydro-4H-imidazol-4-one hydrochloride (**21**)

(Z)-5-(3-(4-Fluorobenzoyloxy)benzylidene)-2-(methylthio)-3,5-dihydro-4H-imidazol-4-one (**41**) (2 mmol, 0.68 g) and 3-(4-methylpiperazin-1-yl)propan-1-amine (2.5 mmol, 0.39 g) were used. Yellow solid. Yield 77 %; mp 229-231 °C. $C_{25}H_{30}FN_5O_2 \cdot 3HCl \cdot H_2O$ MW 578.94. 1H -NMR (DMSO- d_6 , ppm): δ 11.98 (br. s, 1H, NH⁺), 9.32 (s, 1H, N3-H), 7.70 (s, 2H, Ar-2,5-H), 7.56-7.43 (m, 2H, Ar'-2,6-H), 7.28-7.15 (t def., 2H, Ar'-3,5-H), 7.13-7.02 (d def., 2H, Ar-4,6-H), 6.73 (s, 1H, C=CH), 5.13 (s, 2H, O-CH₂), 4.04-2.96 (m, 12H, Pip, N3-CH₂, N3-CH₂-CH₂-CH₂, H₃O⁺), 2.79 (s, 3H, Pip-N-CH₃), 2.01 (s, 2H, N3-CH₂-CH₂). LC/MS±: purity 98.63 % $t_R = 4.03$, (ESI) m/z $[M+H]^+$ 452.42. Anal. calcd for $C_{25}H_{30}FN_5O_2 \cdot 3HCl \cdot H_2O$: C, 51.86; H, 6.11; N, 12.10; found: C, 51.81; H, 5.86; N, 11.95.

(Z)-2-Amino-5-(3-(4-chlorobenzoyloxy)benzylidene)-3-(3-morpholinopropyl)-3,5-dihydro-4H-imidazol-4-one hydrochloride (**22**)

(Z)-5-(3-(4-Chlorobenzoyloxy)benzylidene)-2-(methylthio)-3,5-dihydro-4H-imidazol-4-one (**42**) (3 mmol, 1.08 g) and 3-morpholinopropan-1-amine (3.5 mmol, 0.50 g) were used. Yellow solid. Yield 59 %; mp 185-187 °C. $C_{24}H_{27}ClN_4O_3 \cdot 2HCl \cdot 2.5H_2O$ MW 572.91. 1H -NMR (DMSO- d_6 , ppm): δ 11.44 (br. s, 1H, NH⁺), 9.61 (s, 1H, N3-H), 7.76-7.23 (m, 7H, Ar-2,5,6-H, Ar'-2,3,5,6-H), 7.09-6.90 (m, 1H, Ar-4-H), 6.75-6.51 (d def., 1H, C=CH), 5.30-5.00 (m, 2H, O-CH₂), 4.29-2.73 (m, 12H, Mor, N3-CH₂, N3-CH₂-CH₂-CH₂, H₃O⁺), 2.03 (s, 2H, 3-CH₂-CH₂). LC/MS±: purity 99.03 % $t_R = 5.10$, (ESI) m/z $[M+H]^+$ 455.31. Anal. calcd for $C_{24}H_{27}ClN_4O_3 \cdot 2HCl \cdot 2.5H_2O$: C, 50.31; H, 5.64; N, 9.78; found: C, 50.34; H, 5.51; N, 9.86.

(Z)-2-Amino-5-(3-(4-chlorobenzoyloxy)benzylidene)-3-(3-(4-methylpiperazin-1-yl)propyl)-3,5-dihydro-4H-imidazol-4-one hydrochloride (**23**)

(Z)-5-(3-(4-Chlorobenzoyloxy)benzylidene)-2-(methylthio)-3,5-dihydro-4H-imidazol-4-one (**42**) (4 mmol, 1.30 g) and 3-(4-methylpiperazin-1-yl)propan-1-amine (5.5 mmol, 0.87 g) were used. Cream solid. Yield 87 %; mp 221-223 °C. $C_{25}H_{30}ClN_5O_2 \cdot HCl \cdot 3H_2O$ MW 558.93. 1H -NMR (DMSO- d_6 , ppm): δ 12.07 (s, 1H, NH⁺), 7.67-7.24 (m, 9H, C2-NH₂, Ar-2,5,6-H, Ar'-2,3,5,6-H), 7.09-6.94 (d def., 1H, Ar-4-H), 6.72 (s, 1H, C=CH), 5.24-5.07 (d def., 2H, O-CH₂), 4.29-2.93 (m, 12H, Pip, N3-CH₂, N3-CH₂-CH₂-CH₂, H₃O⁺), 2.78 (s, 3H, Pip-N-CH₃), 2.22-1.92 (m, 2H, N3-CH₂-CH₂). LC/MS±: purity 97.54 % $t_R = 4.33$, (ESI) m/z $[M+H]^+$ 470.40. Anal. calcd for $C_{25}H_{30}ClN_5O_2 \cdot HCl \cdot 3H_2O$: C, 53.76; H, 6.69; N, 12.54; found: C, 53.40; H, 6.82; N, 12.00.

Biological assays

Cell lines

The mouse T-cell lymphoma L5178 cells (PAR) (ECACC Cat. No. 87111908, coming from FDA, Silver Spring, MD, USA) were transfected with pHa MDR1/A retrovirus. The ABCB1-expressing cell line L5178Y (MDR) was elected by the culture of the infected cells with colchicine. The parental mouse L5178 cells (PAR) and the subline L5178Y of human ABCB1-transfected mouse T-cell lymphoma (MDR) were cultured in McCoy's 5A medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% heat-inactivated horse serum (Sigma-Aldrich, St Louis, MO, USA), 200 mM L-glutamine (Sigma-Aldrich, St Louis, MO, USA), and a penicillin-streptomycin (Sigma-Aldrich, St Louis, MO, USA) mixture in concentrations of 100 U/L and 10 mg/L, respectively. The cell lines were incubated in a 5% CO₂, 95% air atmosphere at 37 °C.

Cytotoxicity assay

The cytotoxicity of compounds was examined in PAR and MDR mouse T-lymphoma cell lines, using the MTT assay to calculate the IC₅₀ values as described previously [24]. Mean IC₅₀ values were obtained by best-fitting the dose-dependent inhibition curves in GraphPadPrism5 program (GraphPad Software version 5.00 for Windows, San Diego, CA, USA) from four parallel experiments for each cell line.

Rhodamine 123 accumulation assay

Compounds were tested for their ABCB1 efflux pump inhibiting activity using the rhodamine 123 accumulation assay according to the protocol described formerly [24]. The fluorescence activity ratio (FAR) was estimated based on the following equation, which relates the measured fluorescence values:

$$FAR = \frac{MDR_{treated}/MDR_{control}}{PAR_{treated}/PAR_{control}}, \text{Quotient} = 100 \times \frac{FAR_{compound}}{FAR_{tariquidar}}$$

Studies *in vitro* on potential mechanism of ABCB1 modulation

To determine influence on ATPase activity of 5-arylidenimidazolones, the luminescent Pgp-Glo™ Assay System was used. It was purchased from Promega (Madison, WI, USA). Three repetitions of the assay was performed according to previously published data [28-30]. Compounds **12**, **20**, and **22** (100 μM) were incubated with Pgp membranes for 40 min at 37 °C. Other conditions were the same as described in our previous article [24]. Measurement of luminescence signal was done by a microplate reader EnSpire PerkinElmer (Waltham, MA, USA). GraphPad Prism 8.0.1 software was used for calculation of statistical significances.

Pharmacophore modelling

As the first step, 2D structures of the compounds **4**, **5**, **7-23** were converted into energy minimized 3D structures (OPLS3 force field [37]) using LigPrep (Schrödinger) [38] and the appropriate ionization states at pH = 7.0 ± 2.0 were assigned using Epik (Schrödinger) [39]. Subsequently, application of the Phase module (Schrödinger) [31] enabled to develop the pharmacophore model from the set of investigated, active/inactive compounds under the default settings (conformers generated during the search, not more than 50 conformers per structure, 10 hypotheses generated). The best hypothesis have mapped 100% of input actives and 40% of input inactives.

Lipophilicity estimation using RP-TLC method

The lipophilicity tests were carried out using the RP-TLC method. Mixtures of appropriate amounts of water and an organic modifier (methanol) ranging from 40 to 90% (v/v) in 5% steps were used as mobile phases. The analysis was performed on silica gel 60 RP-18

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F254 plates (14 x 10 cm) (Merck, Darmstadt, Germany). Methanol solutions of the analyzed compounds (in a volume of 10 µl) were applied to the plates in the form of 5 mm bands at intervals of 5 mm and 10 mm from the lower edge and sides of the plates using a Linomat V applicator (Camag, Basel, Switzerland). A vertical chromatography chamber (Sigma-Aldrich, St. Louis, USA) with dimensions of 20×10×18 cm was saturated with the mobile phase for 20 min. The plates were developed to a height of 9.5 cm and then, after drying at room temperature, they were observed under ultraviolet light at 254 and/or 366 nm (UV lamp, Camag, Basel, Switzerland). Each analysis was performed in triplicate and mean RF values (delay factor) were calculated.

Based on the determined RF values, the RM parameters were calculated according to the formula:

$$RM = \log(1/RF - 1)$$

Then, linear correlations between the RM values for each of the tested substances and the concentration of the organic modifier in mobile phases were determined using the Soczewiński-Wachtmeister equation [40]:

$$RM = R_{M0} + aC$$

where C is the concentration of organic solvent (in%) in the mobile phase, and is the slope of the curve, and R_{M0} is the concentration of the organic modifier extrapolated to zero.

Lipophilicity prediction in silico

Computer-aided lipophilicity predictions were performed using the following bioinformatic tools: SwissADME (<http://www.swissadme.ch/>) [32], pkCSM (<http://biosig.unimelb.edu.au/pkcsml/>) [33], molinspiration (<https://molinspiration.com/cgi-bin/properties>) [34], and ALOGPS (<http://www.vcclab.org/web/alogps/>) [35,36]. Compounds 7–23 were drawn in basic forms. From obtained logP results, arithmetic mean and standard deviations were calculated.

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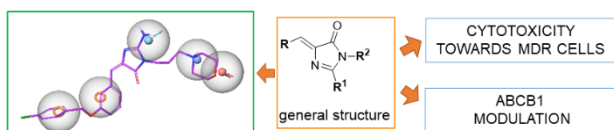
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New 2-amine-5-arylideneimidazolones are promising chemical group for further development with proved selective cytotoxicity towards multidrug resistant cancer cells as well as ABCB1 modulatory properties. The results from biological assays (MTT and rhodamine 123 accumulation) in combination with ABCB1 pharmacophore modeling were discussed in the structure-activity relationship section pointing out the lead structure for further modifications.