Design, synthesis, antitrypanosomal activity, DNA/RNA binding and *in vitro* ADME profiling of novel imidazoline-substituted 2-arylbenzimidazoles

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Abstract

Novel imidazoline benzimidazole derivatives containing diverse substituted phenoxy moieties were synthesized with the aim of evaluating their antitrypanosomal activity, DNA/RNA binding affinity and *in vitro* ADME properties. The presence of the diethylaminoethyl subunit in **18a**–**18c** led to enhanced antitrypanosomal potency, particularly for **18a** and **18c**, which contain unsubstituted and methoxy-substituted phenoxy moieties. They were found to be > 6-fold more potent against African trypanosomes than nifurtimox. Fluorescence and CD spectroscopy, thermal denaturation assays and computational analysis indicated a preference of **18a**–**18c** toward AT-rich DNA and their minor groove binding mode. Replacement of the amidine group with less basic and ionisable nitrogen containing moieties failed to improve membrane permeability of the investigated compounds. Due to structural diversification, the compounds displayed a range of physico-chemical features resulting in variabile *in vitro* ADME properties, leaving space for further optimization of the biological profiles.

Keywords: Imidazoline-substituted benzimidazole, ADME, DNA binding, *Trypanosoma brucei*

1. Introduction

Trypanosomatid protozoan parasites have a significant socioeconomic impact worldwide [1]. Amongst them are parasites of the *Trypanosoma brucei* species complex, which cause human African trypanosomiasis (HAT), also known as sleeping sickness. In the early stage of the disease, parasites invade the blood and lymphatic system, while in the second stage, they penetrate the blood–brain-barrier (BBB) and enter the central nervous system (CNS), leading to coma and death if untreated [2]. The current drugs used to treat HAT, pentamidine, suramin, melarsoprol and nifurtimox-eflornithine combination therapy (NECT) [1], are toxic and not always effective. Drug-resistance, often related to transporters responsible for the drug uptake, has been widely characterised [5,6]. A further limitation of diamidines, which have high therapeutic interest as antiparasitic agents [7], is the requirement for parenteral administration due to poor oral bioavailability resulting from the high pKa of the amidine functional group [8]. Although the active transport of amidines ensures that they reach the site of action, their permanent charge limits membrane permeability and, thus, oral bioavailability.ref However, the permeability and activity of amidines was successfully modulated by lowering the pKa of bisimidazoline compounds with incorporated halogen or nitrogen atoms [8,9]. To improve chemotherapeutic treatment of HAT, the boron-containing small molecule, acoziborole, was developed as an orally active drug candidate that is efficacious in both stages of the disease [10,11].

Various biological targets have been suggested to account for the antiparasitic activity of trypanocidal drugs, including both mitochondrial and nuclear DNA, heat shock protein 70 (Hsp70) [12], microtubules [13], acidocalcisomes [14], trypanosomatid proteasomes [15] and a range of enzymes [16]. Compounds that act as mitochondrial kinetoplast DNA (kDNA) binders have been shown to selectively target AT-rich DNA and to form a complex in the minor groove of the double helix [17–19].

Previous studies by our group [20–22] and others [23–30] have shown that nitrogen heterocycles, such as benzimidazole derivatives, have good antiprotozoal potency. The 5-nitroimidazole derivative, fexinidazole, has been recently developed as an oral treatment of HAT [31–33] and will be a major advance over the current therapy [34]. In addition, triazolo-containing compounds have demonstrated *in vivo* efficacy for trypanosomiasis that is superior to the clinical drug melarsoprol [35,36]. We have designed imidazoline benzimidazole derivatives with substituted phenoxy moieties to modulate physico-chemical and biological properties (Figure 1). Antitrypanosomal potencies and DNA/RNA binding properties of the

compounds were evaluated. Calculated physico-chemical properties were compared with measured *in vitro* ADME properties, microsomal metabolic stability and membrane permeability.

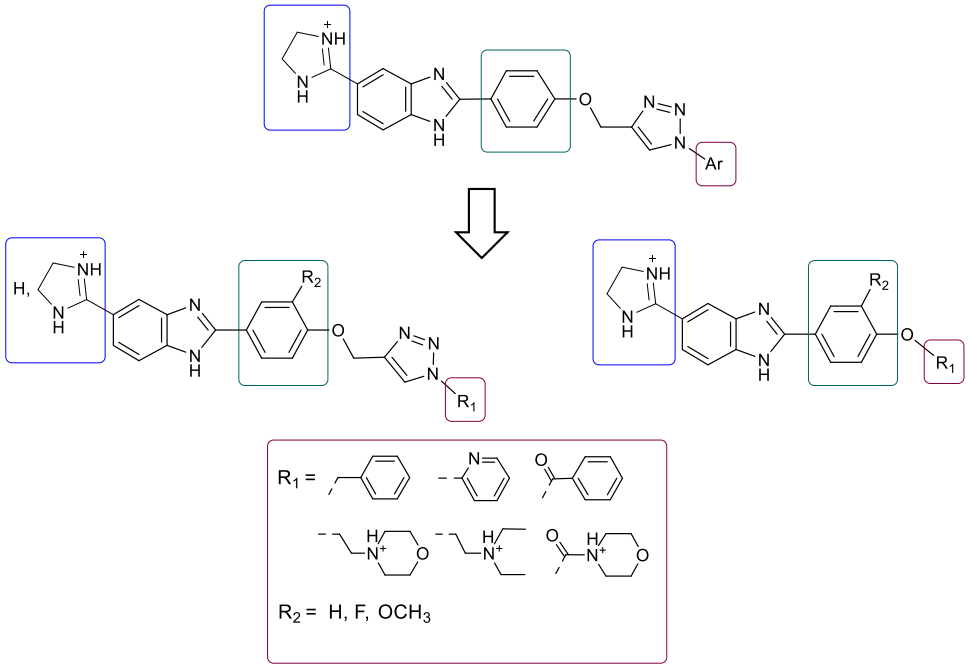
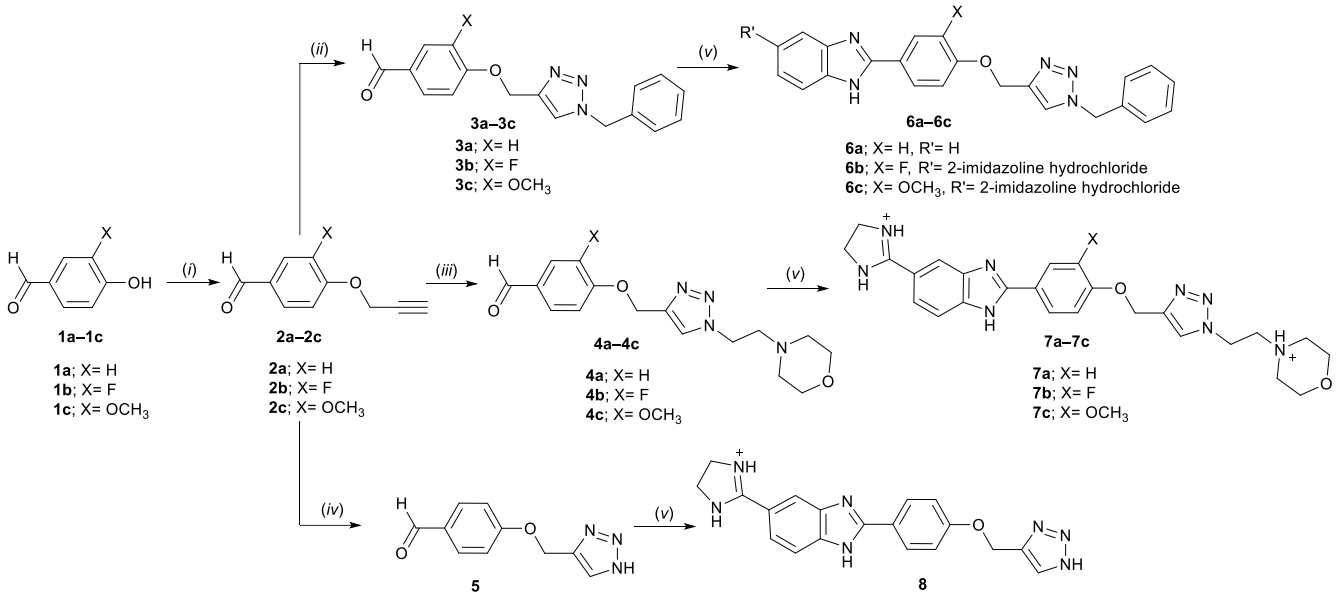


Figure 1. Design of novel imidazoline-substituted benzimidazoles **6a**–**6c**, **7a**–**7c**, **8**, **14a**–**18a**, **14b**–**18b** and **14c**–**18c** with a range of cyclic, aromatic as well as ionisable aromatic and aliphatic subunits.

2. Results and discussion

2.1. Chemistry

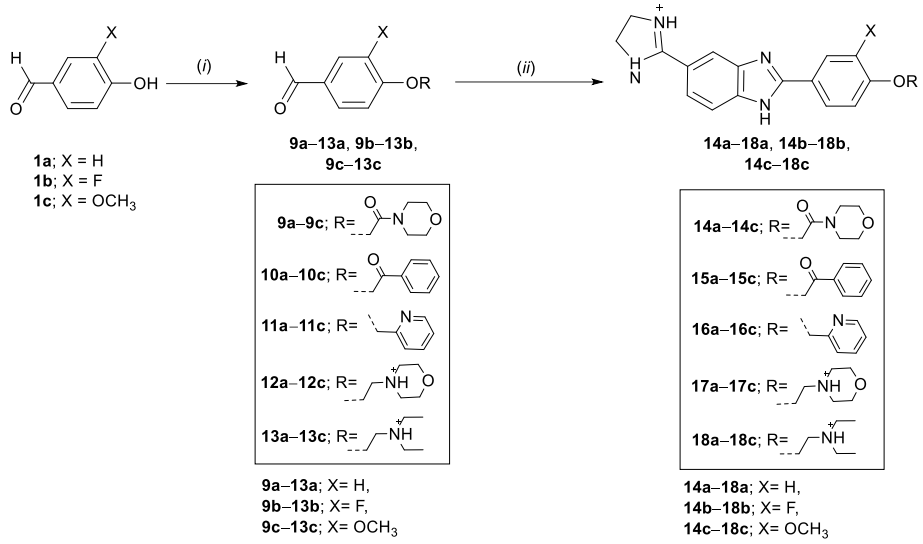
The synthesis of novel 2-aryl substituted benzimidazole derivatives **6a**–**6c**, **7a**–**7c**, **8**, **14a**–**18a**, **14b**–**18b** and **14c**–**18c** was carried out as shown in Scheme 1 and 2. Key intermediates for the synthesis of 1,2,3-triazolyl linked 2-aril benzimidazole derivatives (**6a**–**6c**, **7a**–**7c** and **8**) were prepared by regioselective copper(I) catalysed cycloaddition from *O*-propargylated benzaldehydes (**2a**–**2c**), used as dipolarophiles, and corresponding azides (Scheme 1).



Scheme 1. *Reagents and conditions*: (*i*) propargyl bromide, AcCN, K_2CO_3, reflux 8 h; (*ii*) benzyl chloride, NaN_3, Et_3N, 30 min, rt, $Cu(OAc)_2$, *t*-BuOH : H_2O = 1 : 1, 24 h; (*iii*) $Cu(OAc)_2$, MeOH, reflux, 24 h; (*iv*) CuI, $TMSN_3$, DMF : H_2O = 9 : 1, reflux, 6 h; (*iv*) 4-(imidazolin-2-yl)benzene-1,2-diamine/1,2-phenylendiamine, $NaHSO_3$/*p*-benzoquinone, EtOH, reflux, 8 h.

1-Benzyl-1,2,3-triazole benzaldehydes **3a**–**3c** were synthesized in excellent yield (82–92%) in a *one-pot click* reaction, 1,3-dipole was formed *in situ* using Cu(II) acetate as a catalyst. 1,3-Dipolar cycloaddition of terminal alkynes (**2a**–**2c**) and 2-(4-morpholine)ethyl azide yielded the corresponding 1-ethylmorpholine-1,2,3-triazole benzaldehyde precursors **4a**–**4c**. The ethylmorpholine substituent was introduced to the N-1 position of the 1,2,3-triazole ring to increase solubility of imidazoline-substituted benzimidazole derivatives **7a**–**7c**. To the determine the influence of substituents on the triazole ring on pharmacokinetic properties and trypanosomal activity, non-substituted triazole **5** was prepared with copper(I) iodide and trimethylsilylazide ($TMSN_3$). Condensation of 4-(1,2,3-triazol-1-yl)benzaldehyde derivatives **3b**, **3c**, **4a**–**4c** and **5** with 4-(imidazolin-2-yl)benzene-1,2-diamine and $NaHSO_3$ or *p*-benzoquinone, as an oxidative reagent, afforded the target imidazoline-substituted benzimidazoles (**6b**–**6c**, **7a**–**7c** and **8**). The 4-(imidazolin-2-yl)benzene-1,2-diamine was prepared by the Pinner reaction previously reported in literature [37]. Furthermore, to look into the influence of the imidazoline substituent, unsubstituted benzimidazole derivative **6a** was prepared by cyclization of *o*-phenylene diamine with benzaldehyde **3a** in 94% yield. With the aim of assessing the influence of the triazole moiety on the antitrypanosomal activity and pharmacokinetic properties, morpholinoyl (**14a**–**14c**), benzoyl (**15a**–**15c**), pyridine (**16a**–**16c**), ethylmorpholine (**17a**–**17c**) and diethylaminoethyl (**18a**–**18c**) substituents were introduced to

the phenoxymethylene linker of the imidazoline-substituted benzimidazoles as displayed in Scheme 2. *O*-alkylation of the hydroxybenzaldehydes **1a**–**1c** with the corresponding halides and K_2CO_3 afforded the benzaldehyde precursors **9a**–**13a**, **9b**–**13b** and **9c**–**13c**, which were converted to targeted 2-aryl-5-(2-imidazolinyl)-substituted benzimidazole (**14a**–**18a**, **14b**–**18b** and **14c**–**18c**) hybrids with $NaHSO_3$ or *p*-benzoquinone.

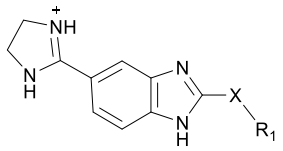


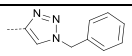
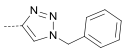
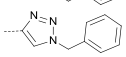
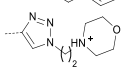
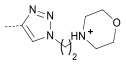
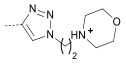
Sheme 2. *Reagents and conditions*: (*i*) RCl/RBr, AcCN, K_2CO_3, reflux, 8 h; (*ii*) 4-(imidazolin-2-yl)benzene-1,2-diamine, $NaHSO_3$/*p*-benzoquinone, EtOH, reflux, 8 h.

2.2. Antitrypanosomal evaluations

Preliminary screening of 2-aryl-substituted benzimidazole derivatives **6a**–**6c**, **7a**–**7c**, **8**, **14a**–**18a**, **14b**–**18b** and **14c**–**18c** against *T. brucei* was performed at different concentrations (100, 10, 1 and 0.1 µg/mL) (Table 1). All compounds displayed 100% growth inhibition at the highest concentration of 100 µg/mL, while at 10 µg/mL, the aromatic phenoxymethylene pyridine **16b** had the lowest inhibition (40%). Best selectivity was observed with **6a**, **7a**, **8** and **18a**−**18c,** which inhibited growth by more than 90% at 1 µg/mL. Thus, from the series of 2-arylbenzimidazoles connected to aromatic and cyclic subunits *via* a 1,2,3-triazole linker, benzimidazole **6a,** which contains benzyl at position N-1 of the triazole and imidazoline-substituted benzimidazoles, **7a** and **7b** with an ethylmorpholine moiety at N-1 of triazole, and **8,** with an unsubstituted triazole ring, showed growth inhibition above 90%. Among the imidazoline benzimidazoles with aromatic and ionisable aliphatic and aromatic subunits directly connected to phenoxymethylene, compounds **18a**–**18c,** with a diethylaminoethyl moiety, exhibited growth inhibition above 90%. At 0.1 µg/mL, only compounds **18a** and **18c** showed any level of inhibition (20%).

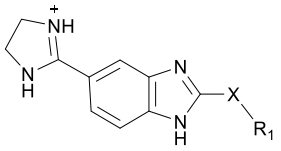
Table 1. Inhibitory properties of compounds **6a**–**6c**, **7a**–**7c**, **8**, **14a**–**18a**, **14b**–**18b** and **14c**–**14c** against cultured bloodstream form *Trypanosoma brucei* (Materials and methods).

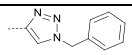
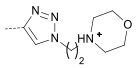
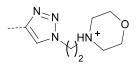


Cmpd	X	R_1	% inhibition 100 µg/mL	% inhibition 10 µg/mL	% inhibition 1 µg/mL	% inhibition 0.1 µg/mL
6a	-PhOCH$_2$-		100	100	90	0
6b	-FPhOCH$_2$-		100	90	0	0
6c	-OCH$_3$PhOCH$_2$-		100	100	0	0
7a	-PhOCH$_2$-		100	100	90	0
7b	-FPhOCH$_2$-		100	100	90	0
7c	-OCH$_3$PhOCH$_2$-		100	100	30	0
8	-PhOCH$_2$-		100	100	90	0
14a	-PhOCH$_2$-		100	100	0	0
14b	-FPhOCH$_2$-		100	100	0	0
14c	-OCH$_3$PhOCH$_2$-		100	80	0	0
15a	-PhOCH$_2$-		100	100	0	0
15b	-FPhOCH$_2$-		100	90	0	0
15c	-OCH$_3$PhOCH$_2$-		100	100	0	0
16a	-PhOCH$_2$-		100	100	0	0
16b	-FPhOCH$_2$-		100	40	0	0
16c	-OCH$_3$PhOCH$_2$-		100	100	0	0
17a	-PhOCH$_2$-		100	100	0	0
17b	-FPhOCH$_2$-		100	80	0	0
17c	-OCH$_3$PhOCH$_2$-		100	90	0	0
18a	-PhOCH$_2$-		100	100	100	20
18b	-FPhOCH$_2$-		100	100	90	0
18c	-OCH$_3$PhOCH$_2$-		100	100	100	20

Based on these results, further antitypanosomal activity evaluations were performed for selected compounds **6a**, **7a**, **7b**, **8**, and **18a**–**18c**, and expressed as the concentration that inhibited growth by 50% (IC_{50}) and 90% (IC_{90}) (Table 2).

Table 2. Antitrypanosomal activity[a] of compounds **6a**, **7a**, **7b**, **8**, and **18a**–**18c** against cultured bloodstream form *Trypanosoma brucei* (Materials and methods).



Cmpd	X	R_1	*T. brucei*		L6 cells	S.I.[c]
			IC_{50} (μM)	IC_{90} (μM)	IC_{50} (μM)	IC_{50} (Tb/L6)
6a	-$PhOCH_2$-		3.75 ± 0.06	-	17.2 ± 0.8	4.6
7a	-$PhOCH_2$-		>4	-	-	-
7b	-$FPhOCH_2$-		>4	-	-	-
8	-$PhOCH_2$-		>4	-	-	-
18a	-$PhOCH_2$-		0.47 ± 0.02	1.82 ± 0.15	>250	>530
18b	-$FPhOCH_2$-		3.67 ± 0.30	-	>250	>68
18c	-$CH_3OPhOCH_2$-		0.71± 0.22	1.47 ± 0.22	>250	>350
Nifurtimox[b]	-	-	4.4 ± 0.7[b]	-		

[a] *In vitro* activity against bloodstream form *T. brucei* expressed as the concentration that inhibited growth by 50% (IC_{50}) and 90% (IC_{90}). Data are the mean of triplicate experiments ± SEM. [b] Taken from ref. [38].
[c] Selectivity index.

Cytotoxicity was assessed using the rat myoblast cell line L6. In terms of trypanosome growth inhibition, imidazoline benzimidazoles **7a**, **7b** and **8**, with the phenoxymethylene-1,2,3-triazole unit, exhibited the lowest activity of the compounds re-tested. Previously, comparing **7a** and **7b** to the corresponding analogues **17a** and **17b**, in which the ethylmorpholine is directly attached to phenoxy core, we had demonstrated that the 1,2,3-triazole linker does improve activity (Table 1). The 2-arylbenzimidazole **8**, which lacks the cationic imidazolino moiety, showed activity (IC_{50} = 3.75 μM) comparable to that of nifurtimox. However, this compound was also relatively cytotoxic (IC_{50} = 17.2 μM) with a selectivity index (S.I.) of 4.6. Introduction of the diethylaminoethyl substituent attached to phenoxymethylene in **18a**–**18c** led to considerably increased and selective activity, particularly **18a** and **18c** that exhibited IC_{50} values in sub-micromolar range (**18a**: IC_{50} = 0.47 μM, IC_{90} = 1.82 μM; **18c**: IC_{50} = 0.73 μM, IC_{90} = 1.47 μM). These compounds were non-cytotoxic (S.I. > 530). Interestingly, addition of the electron-withdrawing fluorine into **18b** resulted in a 6-fold reduction in antitrypanosomal activity compared to non-substituted structural congener **18a**, whereas analogue **18c**, with an electron-donating methoxy group showed comparable activity to **18a**.

2.3. DNA and RNA binding study

Based upon antitrypanosomal activity, compounds **18a**–**18c** were selected for the binding study with DNA and RNA. As previously mentioned, dicationic molecules with antitrypanosomal activity, such as the bis(2-aminoimidazoline) drugs, were found to be DNA minor groove binders, with selectivity toward AT-sequences [18,39]. According to literature data, this selectivity is predominantly responsible for their antitrypanosomal activity [18,40]. Thus, the aim of this study was to further examine DNA and RNA interactions with highly active trypanocides **18a**–**18c**. Evaluated compounds were dissolved in redistilled water. Those solutions were used for measurements in aqueous buffer (pH = 7, sodium cacodylate buffer, I = 0.05 mol dm^{-3}). The UV/Vis spectra (Figures S1-S5, Supporting Information, SI) of buffered solutions of **18a**–**18c** were proportional to their concentrations up to $c = 2 \times 10^{-5}$ mol dm^{-3}, suggesting that these compounds do not aggregate by intermolecular stacking at the experimental conditions used. The absorption maxima and the corresponding molar extinction coefficients (ε) are given in Table S1 (SI). Changes in the UV/Vis spectra of the compounds as the temperature increased to 95 °C were negligible, and the reproducibility of UV/Vis spectra upon cooling back to 25 °C was excellent. The excitation spectra agreed well with the corresponding absorption spectra in the region where emission and excitation spectrum do not overlap (Figures S6-S13, SI). Binding of the compounds to DNA and RNA polynucleotides was monitored with the fluorescence spectroscopy. CD titrations and ΔT_m experiments were used to determine the binding modes (intercalation, groove binding or external binding).

For initial measurements with ds-polynucleotides, we chose *calf thymus* DNA (ctDNA), which represents a classical B-helix, and poly A – poly U as a model for RNA, with a characteristic A-helical structure of a wide and shallow minor groove and a deep and narrow major groove [41]. After acquiring preliminary results with ctDNA, we decided to perform experiments with synthetic polynucleotides, poly(dAdT)$_2$ and poly(dGdC)$_2$, both of which form a classical B-helix, but in the case of the latter, with a minor groove that is sterically hindered by the amino groups of guanine [42]. Polynucleotide addition to compound solutions resulted in an emission increase of **18c**, and fluorescence quenching of **18a** and **18b** (Figure 2, Table 3).

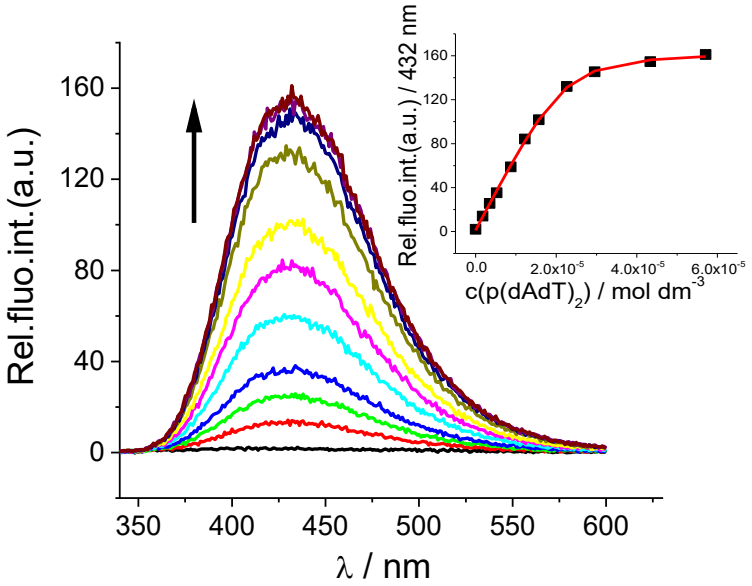


Figure 2. Changes in fluorescence spectrum of **18c** ($c = 1 \times 10^{-6}$ mol dm^{-3}, $\lambda_{exc} = 320$ nm) upon titration with poly(dAdT)$_2$ ($c = 1.7 \times 10^{-6} - 5.7 \times 10^{-5}$ mol dm^{-3}); Inset: dependence of **18c** absorbance at $\lambda = 432$ nm on c(poly(dAdT)$_2$), at pH = 7, sodium cacodylate buffer, $I = 0.05$ mol dm^{-3}.

18a and **18b** showed dual fluorimetric responses in titrations with AT-DNA (poly(dAdT)$_2$). First additions of this polynucleotide induced a decrease in **18a** and **18b** fluorescence, while further aliquots yielded a significant fluorescence increase. Unlike **18a** and **18b**, the emission maximum of **18c** did not shift on its binding to most of studied polynucleotides. Fluorescence changes of **18c** with GC-DNA were too small for accurate calculation of binding constants (Table 3).

Table 3. Binding constants (log*Ks*)[a] and ratios *n*[b] ([bound compound]/[polynucleotide phosphate]) calculated from the fluorescence titrations of **18a**–**18c** with ds-polynucleotides at pH = 7.0 (buffer sodium cacodylate, $I = 0.05$ mol dm^{-3}).

	ctDNA			poly A - poly U			p(dAdT)$_2$			p(dGdC)$_2$		
	log*K*$_s$	*n*	I/I$_0$[d]	log*K*$_s$	*n*	I/I$_0$[d]	log*K*$_s$	*n*	I/I$_0$[d]	log*K*$_s$	*n*	I/I$_0$[d]
18a	5.9	0.2	0.5	5.1	0.2	0.4	5.2	0.1[c]	2.0	5.3	< 0.1	< 0.1
18b	6.0	0.1	0.3	4.9	0.2	0.2	6.4	0.1[c]	1.3	4.5	0.4	< 0.1
18c	6.8	< 0.1	43.4	5.4	< 0.1	23.7	7.1	< 0.1	146	-[e]	-[e]	-[e]

[a] Accuracy of *n* ± 10 - 30%, consequently log*Ks* values vary in the same order of magnitude;

[b] Processing of titration data by means of Scatchard equation [43] gave values of ratio n[bound compound]/[polynucleotide]; correlation coefficients were >0.99 for most of calculated K_a;
[c] In case of **18a** and **18c** with poly(dAdT)$_2$, logK_a values (part of titration experiment where emisssion increased) were re-calculated for fixed n = 0.1 due to a more reliable fit than with free stoichiometry; logK_a values could not be calculated from part of titration where **18a** and **18c** emisssion decreased since these changes were too small for accurate calculation of binding constants;
[d] I_0 – starting fluorescence intensity of **18a**–**18c**; I – fluorescence intensity of **18a**–**18c**/polynucleotide complex calculated by Scatchard equation;
[e] Fluorescence changes of studied compound with polynucleotides were too small for accurate calculation of binding constants.

Interestingly, **18a**–**18c**, that differ only in the phenyl substitution (H vs F vs OCH_3), caused opposite fluorimetric changes on interaction with the studied polynucleotides. While addition of **18a** and **18b** caused a decrease in fluorescence in the presence of DNA/RNA, the addition of any polynucleotides resulted exclusively in a strong emission increase of **18c**, which may be ascribed to an increased electron density in the phenyl ring due to the electron-donating methoxy group (Figures S14-S25, SI).

The binding constants *Ks* and ratios $n_{[\text{bound compound}]/[\text{DNA/RNA}]}$ obtained by processing of fluorimetric titration data with the Scatchard equation [43] are summarized in Table 3. All compounds showed greater affinity toward DNA, especially AT-DNA. In particular, **18c** exhibited AT-DNA selectivity over GC-DNA.

Non-covalent binding of small molecules to ds-polynucleotides usually has certain effects on the thermal stability of helices, thus, giving different melting temperature (T_m) values. Melting temperature depends on both the length and the specific nucleotide sequence composition of the polynucleotide. It can be also influenced by salt concentration and pH. Differences between the T_m value of the free polynucleotide and that obtained in complex with small molecules (ΔT_m value) is an important factor in the characterisation of small molecule-ds-polynucleotide interactions [44]. All studied compounds showed a smaller stabilization effect of ds-RNA (poly A - poly U) compared to ds-DNA (Table 4).

Table 4. The ΔT_m[a] values (°C) of ds-polynucleotides upon addition of ratio $\boldsymbol{r}^b$ = 0.3 and $\boldsymbol{r}^b$ = 0.5 of **18a**–**18c** at pH = 7.0 (sodium cacodylate buffer, I = 0.05 mol dm^{-3}).

	ctDNA	poly A – poly U	poly (dA – dT)$_2$
18a	6.4	4.5	17.9
18b	4.5	2.4	14.7
18c	7.2	2.3	19.3

[a] Error in ΔT_m: ± 0.5 °C;
[b] $\boldsymbol{r}$ = [compound]/[polynucleotide]

Furthermore, all compounds showed a significant but smaller stabilization effect toward mixed DNA basepairs than to AT-DNA. This difference in stabilization effect can be explained by ctDNA composition which contains 58% AT and 42% GC nucleobases (Figure 3) [45].

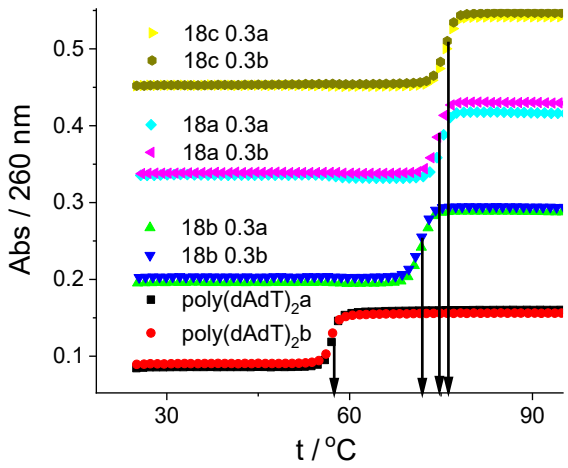


Figure 3. Melting curve of poly(dAdT)$_2$ upon addition of ratio, ***r*** ([compound/ [polynucleotide]) = 0.3 of **18a**–**18c** at pH = 7.0 (buffer sodium cacodylate, I = 0.05 mol dm^{-3}).

Compounds **18a,** without a substituent on the phenyl, and **18c,** with a methoxy-substituted phenyl moiety, showed the best stabilization effect of AT-DNA (Figure 3). Such AT preference is characteristic of small molecules that bind into a minor groove of ds-polynucleotides [19,45]. In the case of ds-RNA, the minor grove is not the probable binding site with these compounds, since it is broad and shallow, in contrast to the deep and narrow minor groove of the ds-DNA [42].

To get insight into conformational changes in the secondary structure of polynucleotides upon small molecule binding, CD spectroscopy was employed [46]. Additional information on the ligand-polynucleotide complexes can be acquired *via* an induced CD (ICD) signal of achiral small molecules when they form complexes with ds-polynucleotides. From this, a mutual orientation of the small molecule and polynucleotide chiral axis can be derived, giving useful information on the modes of interaction [47,48]. The ICD signals observed at $\lambda > 300$ nm can be attributed solely to the studied achiral compounds, since they display UV/Vis spectra in this region while polynucleotides do not (Figures S1-S4, SI).

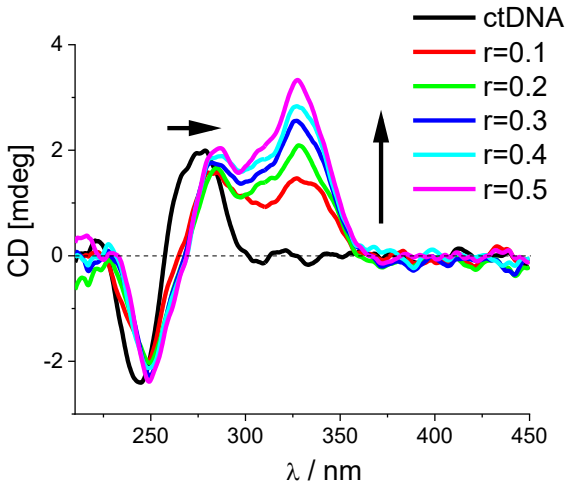


Figure 4. CD titration of poly(dAdT)$_2$ (c = 3.0 × 10^{-5} mol dm^{-3}) with **18a** at molar ratios ***r*** = [compound]/[polynucleotide] (pH = 7.0, buffer sodium cacodylate, I = 0.05 mol dm^{-3}).

Addition of the compounds resulted in a decreased CD spectra of RNA and DNA polynucleotides (Figures S29-S30, SI). Conversely, an increase of CD spectra of poly(dGdC)$_2$ was noticed on addition of all the studied compounds. All compounds exhibited positive induced CD spectra (ICD) with ctDNA and poly(dAdT)$_2$ in the region from 330-340 nm (Figures S29 and S30, SI). Such changes were observed for cationic benzimidazoles and indoles that were found to be minor groove binders [19,48]. The positive ICD spectra, located around 336 nm, were also observed in titrations of poly A – poly U with all compounds studied (Figure S29, SI). Additionally, clear isodichroic points were observed in titrations of **18a** with AT-DNA and **18c** with poly A – poly U, suggesting one dominant interaction mode of these compounds with the DNA/RNA chiral axis [47,48].

A positive ICD band, with an intensity similar or stronger than the CD band of DNA/RNA, strongly supports the minor groove binding to DNA, or the major groove binding to ds-RNA [49]. Thus, it can be concluded that the compounds bind to minor groove of ds-DNA and to major groove of ds-RNA. This is additionally supported by thermal stabilization of ds-polynucleotides (Table 4) and the binding constants ≥ 10 μM (Table 3). Appearance of a dual fluorimetric response in the titrations of **18a** and **18b** with AT-DNA, suggests two different modes of binding, probably monomer binding inside the groove at ratios ***r***[**18a**, **18b**]/[poly(dAdT)$_2$] < 0.2 and formation of lower order aggregates of **18a** and **18b** at ratios ***r*** > 0.2. The intensity of the CD spectrum of poly(dGdC)$_2$ significantly increased upon addition of all compounds and there were no ICD bands at wavelengths longer than 300 nm. Such changes

suggest a non-intercalative mode of binding, probably aggregation along the polynucleotide backbone, most likely inside the hydrophobic major grooves [50].

2.4. Computational analysis of imidazoline-substituted benzimidazoles binding to DNA

To further investigate the results obtained by fluorescence and CD spectroscopy, binding of **18a**–**18c** to the DNA minor groove at the molecular level, as well as their selectivity towards the AT sequences, were investigated by computational methods.

Available X-ray structures of DNA complexes with binders, structurally related to imidazoline-substituted benzimidazoles, were retrieved from the Protein Databank [51–53] and retrieved DNA-ligand complexes and interactions were analysed (pdb codes: 1D30, 1D64, 1DNH, 109D, 1FTD, 1M6F, 1Z8V, 1JTL, 1RMX, 2DND, 2B0K, 2GYX, 442D, 445D, 403D, 458D/459D, 4AH1, 432D, 5T4W, 5LIT, 6BNA, 6EL8). Further computational experiments were carried out using the crystal structure of the DB921-D(CGCGAATTCGCG)2 complex (pdb: 2B0K) and the structure of the DNA duplex d(AAATTT)2 with the potential antiparasitic drug 6XV (pdb: 5LIT). These were used as reference structures of AT-rich DNA complexes, in addition to the crystal structure of the Forkhead domain of human FOXN1 in complex with human ctDNA (6EL8) for selectivity investigation. Structures of inhibitors and interactions with DNA are given in Figure 5.

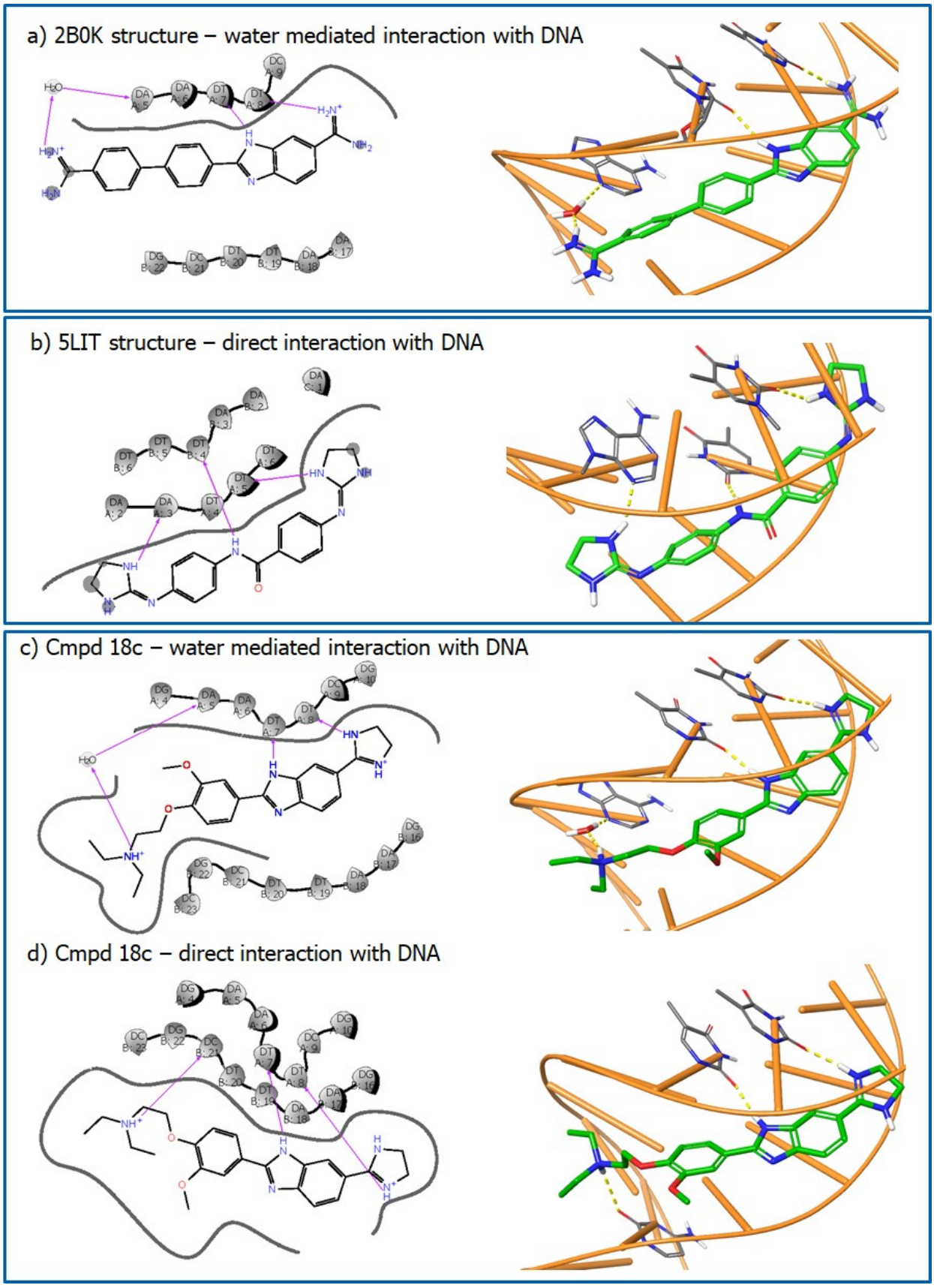


Figure 5. DNA interactions with known ligands (pdb codes: 2B0K and 5LIT) and compound **18c**.

Compounds **18a**–**18c** were docked to the X-ray templates of DNA as described in the Methods section. Both, water mediated and direct binding to the minor groove of the DNA were examined. Complexes were further optimized by the MMGBSA method, and the energy of interactions is shown in Table 5.

Table 5. Ligand ΔG(int)/kcalmol^{-1} with AT-rich DNA and ctDNA.

Cmpd	AT-rich DNA	AT-rich DNA + water	ctDNA	ctDNA + water
2B0K ligand	-80	-84	-68	-73
18c	-100	-75	-84	not forming

Compounds **18a**–**18c** could bind directly as well as through water mediated interactions to compensate the deviations from isohelical structures as shown in Figure 5. Both complexes form three hydrogen-bonds with the DNA bases, with the only difference noticed being for the interaction of the diethylamino group. In the case of water mediated binding, all hydrogen bonds are formed with chain A of the DNA helix, while in the direct complex, the diethylamino group forms a hydrogen bond with the C21 of the chain B. At 2.5Å, this is slightly longer than the other two strong H-bonds of 1.8 and 2.0 Å, respectively.

For the most active compounds shown in the Table 5, deviation from the ideal isohelical structure is around 20°, comparable to a DAPI molecule (pdb: 1D30), and the deviation from the linear geometry is around 30°.

As already shown for a number of minor groove binders [8,53–55], lack of an isohelical structure could be efficiently compensated for by water mediated interactions. In order to investigate which complex would be energetically favourable, docked geometries were re-optimised and free energies of interaction were calculated by the MMGBSA method. We employed molecular mechanics to estimate the strength of interaction and a generalised Born model and solvent accessibility method to implicitly describe solvent effects. Results are shown in Table 5 for the 2B0K ligand, as reference, and compound **18c**. It can be seen, from the calculated free energies, that the 2B0K ligand forms a more stable water mediated complex, while for compound **18c**, direct interactions are more favourable. This is not surprising since the flexible diethylaminoethylene chain can easily adopt a conformation that enables the formation of H-bonds with DNA bases. In addition, molecular dynamic simulations were performed to check the dynamics of ligand-DNA interactions. In the case of 2B0K, two direct and one water mediated hydrogen bond is formed and maintained throughout the whole

simulation time. For the **18c** ligand, in addition to two direct H-bonds, a third H-bond is formed both directly and bridged by a water molecule, increasing the probability of strong interaction. Findings from these structural studies are in agreement with the observed antitrypanosomal activity, since the most active compounds, **18a**–**18c**, possess a flexible diethylaminoethyl group. In contrast, compounds with more rigid and 3D shaped morpholine, pyridine and phenyl moieties exhibit lower activity. Furthermore, compound **18c** binds with higher selectivity to AT-rich DNA in comparison to ctDNA, as shown by calculated free binding energies (Table 5).

To further explore the dynamics of water mediated ligand-DNA interactions, molecular dynamic simulations in water as the solvent were performed for 20 ns at room temperatures to ensure stability of the DNA oligomers [56]. Simulations were carried out for 2B0K and **18c** ligands, and the MMGBSA optimised complex structures were used as starting points. The average number of direct H-bonds during the simulation is in agreement with docking and MMGBSA predictions, while water mediated H-bonds are more dynamic and occur with lower frequency than direct H-bonds, as shown in Figure 6.

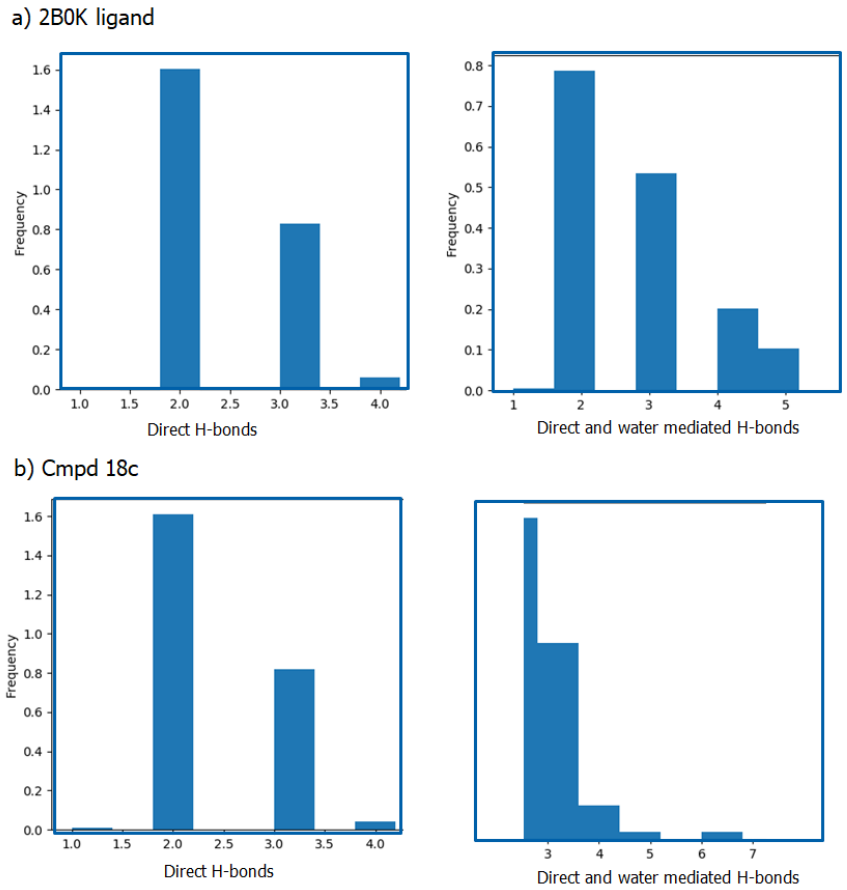


Figure 6. Average number of direct and water mediated H-bonds during MD simulation for a) 2B0K and b) **18c** ligands.

2.5. In vitro ADME profiling

Metabolic stability in mouse liver microsomes and permeability in the MDCKII-hMDR1 assay were determined for synthesized imidazoline-substituted benzimidazole derivatives and the results are shown in Table 6. In addition, lipophilicity, solubility, potential for CYP inhibition and metabolism, binding to plasma proteins and structural parameters were calculated by ACD Percepta software (Table 6). Metabolic studies have shown that majority of compounds have low (< 30% LBF) to moderate clearance (30-70% LBF), while compounds **6a**−**6c** and **15a**−**15c** with phenyl ring have high clearance (> 70% LBF), indicating the impact of the substituent at the triazole and phenoxy unit on metabolic stability. Thus, in the series of compounds with the triazole moiety, imidazoline benzimidazole **8**, with an unsubstituted triazole showed low clearance and high stability in microsomes, whereas ethylmorpholine-substituted analogues **7a**−**7c** and, particularly, the benzyl-substituted derivatives **6a**−**6c**, exhibited reduced microsomal stability. Among the series containing a phenoxy core directly substituted with cyclic, aromatic and aliphatic moieties, morpholinoyl (**14a**–**14c**), methylpyridine (**16a**–**16c**) and diethylaminoethyl (**18a**–**18c**) substitution improved the metabolic stability.

Although compounds were designed to contain one, instead of two amidine moieties, they were all characterized by low membrane permeability (P_{app}(AB) < 2 x 10^{-6} cm/s). High efflux was also measured for compounds **16a**, **16c** and **18a**−**18c**. A major bottleneck to the membrane permeability is the permanently charged amidine moiety. Compound **6a**, which does not contain an amidine group, has a high AB permeability of 12.49 x 10^{-6} cm/s. However, the amidine moiety was shown to be important for the DNA binding and active transport into the parasitic cell, as evidenced by the observed antitrypanosomal activity. Therefore, modulation of amidine basicity will be essential in order to increase membrane permeability and to retain biological activity. pKa values were predicted to range from 11 to 13 for amidine moieties, around 9 for aliphatic amines, and around 7 for morpholine derivatives, which is in agreement with the published data [57–59]. Imidazoline benzimidazoles fall in the good lipophilicity range, with a calculated logP spanning from 1.14 to 3.17, and logD from -1.2 to 1.31. Non-amidino benzimidazole **6a** has logP value of 4.15. Binding to plasma proteins (PBP) is predicted to be lower than 91% for imidazoline benzimidazoles, with ethylmorpholine (**7a**−**7c** and **17a**−**17c**) and diethylaminoethyl (**18a**−**18c**), ensuring an acceptable drug free fraction, while compounds **6a**−**6c** with N-1 benzyl-substituted triazole showed the highest binding (> 99% PBP).

Table 6. Measured metabolic stability in mouse liver microsomes (Pred *in vivo* hep CL) and apparent permeability (P_{app}) in MDCKII-hMDR1 cell assay from apical-to-basolateral (AB) and basolateral-to-apical (BA) side and calculated physicochemical and structural properties.

	Measured properties				Calculated properties									
Cmpd	Pred *in vivo* hep CL [% LBF]	P_{app} (AB) [x10^{-6} cm/s]	P_{app} (BA) [x10^{-6} cm/s]	Efflux ratio	TPSA[a]	AR[b]	RB[c]	HBD[d]	HBA[e]	LogP	LogD	Solubility	pKa	PPB[f] (%)
6a	74.22	12.49	N/A	N/A	68.6	5	6	1	6	4.15	4.15	Highly insoluble	5.2	99.62
6b	84.37	<0.1	0.09	N/A	93.0	5	7	2	8	3.07	1.21	Insoluble	12.6	98.92
6c	79.00	<0.1	<0.1	N/A	102.2	5	8	2	9	2.67	0.81	Insoluble	10.9	98.98
7a	47.87	1.09	1.18	1.09	105.5	4	8	2	10	1.2	-0.7	Soluble	10.9/7.4	88.65
7b	73.42	0.49	0.80	1.64	105.5	4	8	2	10	1.32	-0.58	Soluble	12.6/7.4	89.15
7c	64.14	0.48	0.89	1.86	114.7	4	9	2	11	1.17	-0.73	Very soluble	10.9	89.55
8	<30	0.46	0.84	1.81	103.9	4	5	3	8	1.58	-0.26	Slightly soluble	11.0	91.08
14a	<30	1.06	1.53	1.44	91.8	3	5	2	8	1.14	-0.72	Slightly soluble	10.9	92.85
14b	34.32	0.86	0.99	1.16	91.8	3	5	2	8	1.27	-0.59	Insoluble	12.6	93.58
14c	<30	1.76	1.38	0.78	101.1	3	6	2	9	0.98	-0.88	Slightly soluble	10.9	88.31
15a	87.06	<0.1	0.11	N/A	79.4	4	6	2	6	3.09	1.23	Insoluble	10.9	99.2
15b	92.59	<0.1	<0.1	N/A	79.4	4	6	2	6	3.17	1.31	Insoluble	12.6	98.15
15c	90.38	<0.1	N/A	N/A	88.6	4	7	2	7	3.08	1.22	Insoluble	10.9	98.63
16a	<30	0.07	0.51	7.82	75.2	4	5	2	6	2.62	0.76	Insoluble	12.7	97.68
16b	<30	<0.1	0.2	>2.4	75.2	4	5	2	6	2.7	0.84	Insoluble	12.6	96.71
16c	<30	0.12	0.52	4.22	84.4	4	6	2	7	2.24	0.38	Insoluble	12.7	96.81
17a	66.97	0.49	0.69	1.40	74.8	3	6	2	7	1.81	-0.09	Soluble	12.7/7.1	82.86
17b	65.49	0.49	0.84	1.70	74.8	3	6	2	7	1.86	-0.03	Soluble	12.5/7.0	86.93
17c	47.79	0.64	0.67	1.04	84.0	3	7	2	8	1.66	-0.25	Soluble	12.7/7.0	82.8
18a	<30	0.27	0.63	2.38	65.5	3	8	2	6	2.99	-0.71	Soluble	12.5/8.9	90.82
18b	<30	0.17	0.49	2.92	65.5	3	8	2	6	2.87	-0.88	Soluble	12.7	89.63
18c	<30	<0.1	0.50	>5.0	74.8	3	9	2	7	2.57	-1.20	Slightly soluble	12.7/9.0	89.41

[a] total polar surface area; [b] number of aromatic rings; [c] number of roratable bonds; [d] number of hydrogen bond donors; [e] number of hydrogen bond acceptors; [f] plasma protein binding.

Compounds containing ionisable groups (**7a**−**7c**, **17a**−**17c** and **18a**−**18c**) are predicted to have good water solubility. Overall, the physico-chemical and *in vitro* ADME properties could be favourably modulated within the current chemical series, with exception of a consistently low membrane permeability.

3. Conclusions

The imidazoline-substituted benzimidazoles with a range of cyclic, aromatic and ionisable aromatic and aliphatic substituents to a phenoxy central core were synthesized and evaluated for their antitrypanosomal activity. Generally, the type of substituents had profound effects on antiprotozoal activities. While a triazole spacer between the phenoxymethylene and the ethylmorpholine (in **7a** and **7b**), and the benzyl moiety (in **6a**), led to improved and rather unselective activity, the diethylaminoethyl directly attached to phenoxy in **18a**–**18c** significantly increased the potency with IC_{50} values in the range of 0.47 to 3.67 μM. Furthermore, introduction of an electron-withdrawing fluorine (**18b**) resulted in decreased growth inhibition, while non-substituted and electron-donating methoxy-substituted phenoxy moieties (**18a** and **18c**) showed comparable antitrypanosomal activity.

Fluorescence and CD spectroscopy, as well as the thermal denaturation assay showed large stabilization effects of AT-DNA, high binding affinities and large positive ICD spectra for **18a**–**18c** that point to their preference toward AT-rich DNA sequences and minor groove binding mode. These results were supported by computational analysis, which showed that compounds **18a**–**18c** with a conformationally unrestricted diethylaminoethyl chain, in comparison to the rigid analogues with cyclic and aromatic moieties, have stronger interaction to DNA and can bind with higher selectivity to AT-rich DNA, in comparison to ctDNA.

In vitro ADME profiling showed that the imidazoline-substituted benzimidazoles with ionisable ethylmorpholine (**7a**−**7c** and **17a**−**17c**) and diethylaminoethyl (**18a**–**18c**) units have favourable ADME properties. However, the low membrane permeability of amidine benzimidazoles suggests further optimization is required in order to modulate amidine basicity [8,9,60] and, therefore, improve permeability and oral bioactivity potential.

4. Material and methods

4.1. General

All the solvents and chemicals were purchased from Aldrich and Acros. Thin layer chromatography was performed on pre-coated Merck silica gel 60F-254 plates, while glass

column slurry-packed under gravity with silica gel (Fluka, 0.063–0.2 mm) was employed for column chromatography. Melting points of compounds were determined using Kofler micro hot-stage. ^{1}H and ^{13}C NMR spectra were recorded on a Varian Gemini 300 (300 and 75 MHz) or Varian Gemini 600 (600 and 150 MHz). All data were recorded in dimethyl sulfoxide (DMSO-d_6) at 298 K. Chemical shifts were referenced to the residual solvent signal of DMSO at δ 2.50 ppm for ^{1}H and δ 39.50 ppm for ^{13}C. Individual resonances were assigned on the basis of their chemical shifts, signal intensities, multiplicity of resonances, H–H coupling constants and with the use of a set of 2D experiments.

4.2. Experimental procedures for the preparation of compounds

4-(Prop-2-ynyloxy)benzaldehyde (**2a**) [61], 3-methoxy-4-(prop-2-ynyloxy) benzaldehyde (**2c**) [62], 4-(imidazolin-2-yl)benzene-1,2-diamine [37], 4-(2-azidoethyl)morpholine [63] were prepared according to known procedure, while compounds 4-(1,2,3-triazol-4-yl)methoxy)benzaldehyde (**3a**) [64], 4-[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methoxy]-3-methoxybenzaldehyde **(3c**) [65]**,** 4-(2-morpholino-2-oxoethoxy)benzaldehyde (**9a**) [66], 3-methoxy-4-(2-morpholino-2-oxoethoxy)benzaldehyde (**9c**) [67], 4-(2-oxo-2-phenylethoxy)benzaldehyde (**10a**) [68], 3-methoxy-4-(2-oxo-2-phenylethoxy)benzaldehyde (**10c**) [69], 4-(pyridin-2-yl-methoxy)benzaldehyde (**11a**) [70], 3-fluoro-4-(pyridin-2-yl-methoxy)benzaldehyde (**11b**) [70], 3-methoxy-4-(piridin-2-yl-methoxy)benzaldehyde (**11c**) [71], 4-(2-morpholinoethoxy)benzaldehyde (**12a**) [72], 3-fluoro-4-(2-morpholinoethoxy)benzaldehyde (**12b**) [73]**,** 3-methoxy-4-(2-morpholinoethoxy)benzaldehyde **(12c)** [74]**,** 4-(2-(diethylamino)ethoxy)benzaldehyde (**13a**) [75], 4-(2-(diethylamino)ethoxy)-3-methoxybenzaldehyde (**13c**) [76], were synthesized according to modified procedures given in the literature.

*Preparation of 3-fluoro-4-(prop-2-yn-1-yloxy)benzaldehyde (**2b**)*

To a solution 3-fluoro-4-hydroxybenzaldehyde (5.0 g, 35.7 mmol) in dry ethanol (8-10 mL) K_2CO_3 (1.2 eq.) was added and the mixture was stirred for 30 min followed by addition of and propargyl bromide (1.2 eq.). The reaction mixture was stirred overnight at reflux temperature. The course of the reaction was monitored by TLC. Upon completion of the reaction, the solvent was evaporated under reduced pressure and the crude residue was purified by column chromatography with CH_2Cl_2. Compound **2b** was obtained as yellow solid (4.5 g, 66%, m.p. = 111–115 °C). ^{1}H NMR (600 MHz, DMSO-d_6) (δ/ppm): 9.88 (1H, d, J = 2.0 Hz, CHO), 7.80

(1H, dd, *J* = 8.4, 0.9 Hz, Ph), 7.73 (1H, dd, *J* = 11.3, 1.9 Hz, Ph), 7.45 (1H, t, *J* = 8.3 Hz, Ph), 5.04 (2H, d, *J* = 2.4 Hz, CH_2), 3.70 (1H, t, *J* = 2.4 Hz, CCH). ^{13}C NMR (75 MHz, DMSO) δ 190.8; 190.8 (d, J_{CF} = 1.8 Hz, CHO), 153.4; 150.1 (d, J_{CF} = 247.6 Hz, Ph-q), 150.2 (Ph-q), 130.4; 130.3 (d, J_{CF} = 5.1 Hz, Ph-q), 128.0; 127.9 (d, J_{CF} = 3.1 Hz, Ph), 115.6; 115.4 (d, J_{CF} = 18.4 Hz, Ph), 115.2; 115.2 (d, J_{CF} = 1.3 Hz, Ph), 79.5 (CCH), 78.0 (CCH), 56.7 (OCH_2).

*4.2.1. General procedure for synthesis of N-1-benzyl-1,2,3-triazole benzaldehydes **3a–3c***

The reaction mixture benzyl chloride (1.1 eq.), NaN_3 (0.9 eq.) and Et_3N (1.3 eq.) was dissolved in *t*-BuOH : H_2O = 1 : 1 (4 mL) and stirred for 30 min. The corresponding terminal alkyne (**2a**–**2c**) and $Cu(OAc)_2$ (0.05 eq) were added. The reaction mixture was stirred overnight at room temperature. The solvent was removed under reduced pressure and purified by column chromatography with CH_2Cl_2.

4-[(1-Benzyl-1*H*-1,2,3-triazol-4-yl)methoxy]-3-fluorobenzaldehyde (3b)

According to the general procedure from compound **2b** (250 mg, 1.40 mmol) compound **3b** was prepared as colourless oil (378.7 mg, 87%). ^{1}H NMR (300 MHz, DMSO-d_6) (δ/ppm): 9.87 (1H, d, *J* = 2.1 Hz, 1H), 8.35 (1H, s, H5'), 7.81–7.77 (1H, m, Ph), 7,70 (1H, dd, *J* = 11.3, 1.9 Hz, Ph), 7.60 (1H, t, *J* = 8.3 Hz, Ph), 7.39–7.29 (5H, m, Ph), 5.63 (2H, s, CH_2), 5.37 (2H, s, CH_2). ^{13}C NMR (75 MHz, DMSO-d_6) (δ/ppm): 191.0; 190.9 (d, J_{CF} = 1.7 Hz, CHO), 153.4; 150.1 (d, J_{CF} = 247.1 Hz, Ph-q) 151.2; 151.1 (d, J_{CF} = 10.9 Hz, Ph-q), 141.9 (C4'), 135.9 (Ph-q), 130.0; 130.0 (d, J_{CF} = 5.0 Hz, Ph-q), 128.9 (Ph), 128.3 (Ph), 128.0 (Ph), 125.3 (C5'), 115.5; 115.3 (d, J_{CF} = 18.3 Hz, Ph), 115.2; 115.1 (d, J_{CF} = 1.5 Hz, Ph), 62.3 (OCH_2), 53.0 (CH_2).

4-[(1-Benzyl-1*H*-1,2,3-triazol-4-yl)methoxy]-3-methoxybenzaldehyde (3c)

According to the general procedure from compound **2c** (250 mg, 1.31 mmol) compound **3c** was prepared as colourless oil (345.6 mg; 82 %). ^{1}H NMR (600 MHz, DMSO-d_6) (δ/ppm): 9.85 (1H, s, CHO), 8.31 (1H, s, H5'), 7.55 (1H, dd, *J* = 8.3, 1.9 Hz, Ph), 7.41–7.31 (7H, m, Ph), 5.62 (2H, s, CH_2), 5.26 (2H, s, CH_2), 3.80 (3H, s, OCH_3). ^{13}C NMR (151 MHz, DMSO-d_6) (δ/ppm): 191.4 (CHO), 152.8 (Ph-q), 149.4 (Ph-q), 142.3 (C4'), 135.9 (Ph-q), 123.0 (Ph-q), 128.8 (Ph), 128.2 (Ph), 128.0 (Ph), 125.7 (Ph), 125.0 (C5'), 112.8 (Ph), 109.9 (Ph), 61.8 (OCH_2), 55.5 (CH_3), 52.9 (CH_2).

*4.2.2. General procedure for the synthesis of **4a–4c***

4-(2-Chloroethyl)morpholine 156 (3.0 g; 16.12 mmol) was dissolved in water (16.12 mL) and NaN_3 (3.15 g; 48.37 mmol) was added. The reaction mixture was stirred at 80 °C for 16 h. After completion of the reaction, the mixture was cooled and the pH was adjusted to 10 using KOH. The mixture was extracted with diethyl ether (3 × 50 mL). The organic layer was dried over anhydrous MgS, filtered, and the filtrate was evaporated under reduced pressure at room temperature. The unstable azide (600 mg, 3.84 mmol) was immediately used for the click reaction with the corresponding *O*-propargylated benzaldehyde **2a**–**2c** (3.84 mmol) and $Cu(OAc)_2$ (34.9 mg; 0.19 mmol) in methanol (4 mL). The reaction mixture was stirred at reflux temperature overnight. The course of the reaction was monitored by TLC. Upon completion of the reaction, the solvent was evaporated under reduced pressure and the residue was purified by column chromatography on eluent CH_2Cl_2 : CH_3OH = 50 : 1.

4-[(1-(2-Morpholinoethyl)-1*H*-1,2,3-triazol-4-yl]methoxy}benzaldehyde (4a)

According to the general procedure from compound **2a** (615.06 mg, 3.84 mmol) compound **4a** was prepared as colourless crystals (1.01 g, 83%, m.p. = 109–113 °C). ^{1}H NMR (300 MHz, DMSO-d_6) (δ/ppm): 9.88 (1H, s, CHO), 8.26 (1H, s, H5'), 7.88 (2H, d, J = 8.8 Hz, Ph), 7.24 (2H, d, J = 8.7 Hz, Ph), 5.29 (2H, s, CH_2), 4.50 (2H, t, J = 6.3 Hz, CH_2CH_2), 3.55–3.47 (4H, m, $CH_{2\text{-morpholine}}$), 2.73 (2H, t, J = 6.3 Hz, CH_2CH_2), 2.44–2.37 (4H, m, $CH_{2\text{-morpholine}}$). ^{13}C NMR (151 MHz, DMSO-d_6) (δ/ppm): 191.3 (CHO), 162.9 (Ph-q), 141.7 (C4'), 131.7 (Ph), 129.9 (Ph-q), 125.1 (C5'), 115.2 (Ph), 66.1 ($CH_{2\text{-morpholine}}$), 61.5 (OCH_2), 57.2 (CH_2CH_2), 52.9 ($CH_{2\text{-morpholine}}$), 46.6 (CH_2CH_2).

3-Fluoro-4-{[1-(2-morpholinoethyl)-1*H*-1,2,3-triazol-4-yl]methoxy}benzaldehyde (4b)

According to the general procedure from compound **2b** (684.13 mg, 3.84 mmol) compound **4b** was prepared as yellow crystals (1.03 g, 80%, m.p. = 89–93°C). ^{1}H NMR (300 MHz, DMSO-d_6) (δ/ppm): 9.87 (1H, d, J = 2.0 Hz, CHO), 8.29 (1H, s, H5'), 7.79 (1H, d, J = 8.5 Hz, Ph), 7.70 (1H, dd, J = 11.3, 1.8 Hz, Ph), 7.61 (1H, t, J = 8.3 Hz, Ph), 5.38 (2H, s, CH_2), 4.50 (2H, t, J = 6.2 Hz, CH_2CH_2), 3.57–3.42 (4H, m, $CH_{2\text{-morpholine}}$), 2.73 (2H, t, J = 6.2 Hz, CH_2CH_2), 2.44–2.32 (4H, m, $CH_{2\text{-morpholine}}$). ^{13}C NMR (75 MHz, DMSO-d_6) (δ/ppm): 190.8; 190.8 (d, J_{CF} = 1.7 Hz, CHO), 153.3; 150.0 (d, J_{CF} = 247.0 Hz, Ph-q), 151.2; 151.0 (d, J_{CF} = 10.8 Hz, Ph-q), 141.2 (C4'), 129.9; 129.9 (d, J_{CF} = 5.0 Hz, Ph-q), 128.2; 128.1 (d, J_{CF} = 3.0 Hz, Ph), 125.5 (C5'), 115.4; 115.2 (d, J_{CF} = 18.3 Hz, Ph), 115.1; 115.1 (d, J_{CF} = 1.5 Hz, Ph), 66.1 ($CH_{2\text{-morpholine}}$), 62.3 (OCH_2), 57.3 (CH_2CH_2), 52.9 ($CH_{2\text{-morpholine}}$), 46.5 (CH_2CH_2).

3-Methoxy-4-{[1-(2-morpholinoethyl)-1*H*-1,2,3-triazol-4-yl]methoxy}benzaldehyde (4c)

According to the general procedure from compound **2c** (729.83 mg; 3.84 mmol) compound **4c** was prepared as white solid (1.3 g, 98%, m.p. = 142–146 °C). ^{1}H NMR (300 MHz, DMSO-d_6) (δ/ppm): 9.85 (1H, s, CHO), 8.25 (1H, s, H5'), 7.56 (1H, dd, J = 8.2, 1.8 Hz, Ph), 7.45–7.33 (2H, m, Ph), 5.27 (2H, s, CH_2), 4.50 (2H, t, J = 6.3 Hz, CH_2CH_2), 3.81 (3H, s, OCH_3), 3.54–3.49 (4H, m, $CH_{2\text{-morpholine}}$), 2.74 (2H, t, J = 6.3 Hz, CH_2CH_2), 2.44–2.37 (4H, m, $CH_{2\text{-morpholine}}$). ^{13}C NMR (75 MHz, DMSO-d_6) (δ/ppm): 191.3 (CHO), 152.8 (Ph-q), 149.3 (C4'), 141.7 (Ph-q), 129.9 (Ph-q), 125.7 (Ph), 125.3 (C5'), 112.6 (Ph), 109.7 (Ph), 66.0 ($CH_{2\text{-morpholine}}$), 61.7 (OCH_2), 57.3 (CH_2CH_2), 55.5 (OCH_3), 52.9 ($CH_{2\text{-morpholine}}$), 46.5 (CH_2CH_2).

*4.2.3. Preparation of 4-[(1H-1,2,3-triazol-4-yl)methoxy]benzaldehyde (**5**)*

Compound **2a** (400 mg, 2.30 mmol) was dissolved in DMF : MeOH = 9 : 1 (4.60 mL), then $TMSN_3$ (0.45 mL, 3.44 mmol) and CuI (21.9 mg, 0.12 mmol) were added to the reaction mixture and stirred overnight at 100 °C. After completion the reaction, mixture was evaporated to dryness and the crude residue was purified by column chromatography (CH_2Cl_2 : CH_3OH = 100 : 1). Compound **5** was obtained as white solid (355.8 mg, 76%, m.p. = 120–123 °C). ^{1}H NMR (300 MHz, DMSO-d_6) (δ/ppm): 15.13 (1H, bs, NH), 9.88 (1H, s, CHO), 8.09–7.99 (1H, m, H5'), 7.88 (2H, d, J = 8.8 Hz, Ph), 7.24 (2H, d, J = 8.7 Hz, Ph), 5.32 (2H, s, CH_2). ^{13}C NMR (75 MHz, DMSO-d_6) (δ/ppm): 191.4 (CHO), 162.9 (Ph-q), 131.8 (Ph), 129.9 (Ph-q), 128.0 (C5'), 115.3 (Ph), 61.2 (CH_2).

*4.2.4. General procedure for the synthesis of benzimidazole derivatives **6a**–**6c** and **8***

The reaction mixture of 4-triazolylbenzaldehyde derivative (**3a**–**3c**, **5**) the corresponding *o*-phenylenediamine and 40% $NaHSO_3$ (aq) was dissolved in EtOH (15 mL) and stirred under reflux for 6–8 h. After completion of the reaction, $NaHSO_3$ was filtered and the reaction mixture was evaporated to dryness. Water was added (5 mL) and the mixture was stirred overnight and filtered. The crude residue was dissolved in HCl saturated EtOH (8–10 mL) and stirred for 4 h. Addition of ether resulted in precipitation of products **6a**–**6c** and **8**. Solid was collected by filtration, washed with anhydrous ether, and dried under vacuum.

2-{4-[(1-Benzyl-1*H*-1,2,3-triazol-4-yl)methoxy]phenyl}-1*H*-benzo[*d*]imidazole (6a)

Compound **6a** was prepared using the above described method from **3a** and 1,2-phenylenediamine (55.30 mg, 0.51 mmol) to obtain **6a** as yellow solid (182.3 mg, 94%, m.p. > 250 °C). ^{1}H NMR (600 MHz, DMSO-d_6) (δ/ppm): 8.32 (1H, s, H5'), 8.11 (2H, d, J = 8.8 Hz, Ph), 7.59–7.55 (2H, m, H4; H7), 7.40–7.36 (2H, m, H5; H6), 7.36–7.31 (3H, m, Ph), 7.24–7.17 (4H, m, Ph), 5.63 (2H, s, OCH_2), 5.24 (2H, s, CH_2). ^{13}C NMR (75 MHz, DMSO-d_6) (δ/ppm): 159.4 (Ph-q), 142.7 (C4'), 135.9 (C3/C7a), 128.7 (Ph), 128.1 (Ph), 128.0 (Ph), 127.9 (Ph), 124.7 (C5'), 122.6 (C2), 121.9 (C5/C6), 115.1 (C4/C7), 61.2 (OCH_2), 52.8 (CH_2). Anal. calcd. for $C_{23}H_{19}N_5O$ (M_r = 381.43): C 72.42, H 5.02, N 18.36; found: C 72.16, H 4.81, N 18.63.

2-{4-[(1-Benzyl-1*H*-1,2,3-triazol-4-yl)methoxy]-3-fluorophenyl}-5-(imidazolin-2-yl)-1*H*-benzo[*d*]imidazole hydrochloride (6b)

Compound **6b** was prepared using the above described method from **3b** (150 mg, 0.48 mmol) and 4-(imidazolin-2-yl)benzene-1,2-diamine (110.2 mg, 0.48 mmol) to obtain **6b** as white solid (79.5 mg, 35%, m.p. = 200–203 °C). ^{1}H NMR (600 MHz, DMSO-d_6) (δ/ppm): 10.83 (2H, s, CNH), 8.45 (1H, s, H4), 8.38 (1H, s, H5'), 7.99–7.96 (2H, m, Ph), 7.98 (1H, d, J = 8.5 Hz, H7), 7.88 (1H, d, J = 8.6 Hz, H6), 7.65 (1H, t, J = 8.6 Hz, Ph), 7.40–7.36 (2H, m, Ph), 7.35–7.32 (3H, m, Ph), 5.64 (2H, s, OCH_2), 5.37 (2H, s, CH_2), 4.02 (4H, s, $\underline{CH_2CH_2}$). ^{13}C NMR (75 MHz, DMSO-d_6) (δ/ppm): 165.0 (CNH), 153.1; 149.9 (d, J_{CF} = 244.9 Hz, Ph-q), 152.5; 152.5 (d, J_{CF} = 2.4 Hz, C2), 148.8; 148.7 (d, J_{CF} = 10.3 Hz, Ph-q), 142.0 (C4'), 135.9 (C7a/C3a), 128.8 (Ph), 128.2 (Ph), 128.0 (Ph), 125.2 (Ph), 123.6 (C5'), 116.6 (C5), 115.8 (C4/C7), 115.4, 115.1 (d, J_{CF} = 20.9 Hz, Ph), 62.2 (OCH_2), 52.9 (CH_2), 44.3 ($\underline{CH_2CH_2}$). Anal. calcd. for $C_{26}H_{22}FN_7O \times HCl \times 0.5H_2O$ (M_r = 512.97): C 60.88, H 4.72, N 19.11; found: C 60.63, H 4.41, N 19.22.

2-{4-[(1-Benzyl-1*H*-1,2,3-triazol-4-yl)methoxy]-3-methoxyphenyl}-5-(imidazolin-2-yl)-1*H*-benzo[*d*]imidazole hydrochloride (6c)

Compound **6c** was prepared using the above described method from **3c** (150 mg, 0.46 mmol) and 4-(imidazolin-2-yl)benzene-1,2-diamine (106.1 mg, 0.46 mmol) to obtain **6c** as white solid (179.4 mg, 81%, m.p. = 197–200 °C). ^{1}H NMR (600 MHz, DMSO-d_6) (δ/ppm): 10.88 (2H, s, CNH), 8.46 (1H, s, H4), 8.35 (1H, s, H5'), 8.16 (1H, s, Ph), 8.08 (1H, d, J = 8.3 Hz, H6), 8.02 (1H, d, J = 8.3 Hz, H7), 7.92 (1H, d, J = 8.5 Hz, Ph), 7.45 (1H, d, J = 8.6 Hz, Ph), 7.40–7.29 (5H, m, Ph), 5.63 (2H, s, OCH_2), 5.28 (2H, s, CH_2), 4.03 (4H, s, $\underline{CH_2CH_2}$), 3.90 (3H, s, OCH_3). ^{13}C NMR (75 MHz, DMSO-d_6) (δ/ppm): 165.2 (CNH), 153.6 (C2), 151.0 (Ph-q), 149.5 (Ph-q), 142.6 (C4'), 139.6 (C3a/C7a), 136.1 (Ph-q), 129.0 (Ph), 128.5 (Ph), 128.2 (Ph), 125.3 (C6),

124.0 (C5'), 121.5 (Ph), 118.8 (Ph-q), 117.2 (C5), 115.8 (C4), 114.9 (C7), 113.7 (Ph), 111.1 (Ph), 61.9 (OCH_2), 56.1 (OCH_3), 53.1 (CH_2), 44.6 ($\underline{CH_2CH_2}$). Anal. calcd. for $C_{27}H_{25}N_7O_2 \times HCl \times H_2O$ (M_r = 534.01): C 60.73, H 5.28, N 18.36; found: C 60.81, H 5.20 N 18.39.

5-(Imidazolin-2-yl)-2-{4-[(1*H*-1,2,3-triazol-4-yl)methoxy]phenyl}-1*H*-benzo[*d*]imidazole hydrochloride (8)

Compound **8** was prepared using the above described method from **5** (150 mg, 0.74 mmol) and 4-(imidazolin-2-yl)benzene-1,2-diamine (168.83 mg, 0.74 mmol) to obtain **8** as white solid (251.5 mg, 94%, m.p. = 229–232 °C). ^{1}H NMR (300 MHz, DMSO-d_6) (δ/ppm): 10.90 (2H, s, CNH), 8.47 (1H, s, H5'), 8.43 (2H, d, J = 8.9 Hz, Ph), 8.07–8.01 (2H, m, H4; H6), 7.94 (1H, d, J = 8.6 Hz, H7), 7.35 (2H, d, J = 8.9 Hz, Ph), 5.35 (2H, s, CH_2), 4.03 (4H, s, $\underline{CH_2CH_2}$). ^{13}C NMR (151 MHz, DMSO-d_6) (δ/ppm): 164.7 (CNH), 161.6 (C2), 152.7 (Ph-q), 130.1 (Ph), 124.4 (C5'), 117.6 (C4), 115.7 (Ph), 115.5 (C7), 114.6 (C6), 61.2 (CH_2), 44.3 ($\underline{CH_2CH_2}$). Anal. calcd. for $C_{19}H_{17}N_7O \times HCl \times 1.5H_2O$ (M_r = 422.87): C 53.97, H 5.01, N 23.19; found: C 53.61, H 4.77, N 23.43.

*4.2.5. General procedure for the synthesis of benzimidazole derivatives **7a**–**7c***

The reaction mixture of the corresponding 4-triazolylbenzaldehyde derivative (**4a**–**4c**), 4-(imidazolin-2-yl)benzene-1,2-diamine (1 eq) and *p*-benzoquinone (1 eq) was dissolved in EtOH (15 mL) and stirred under reflux for 6–8 h. Addition of ether resulted in precipitation of products **7a**–**7c**. Solid was collected by filtration, washed with anhydrous ether, and dried under vacuum.

5-(Imidazolin-2-yl)-2-{4-[(1-(2-morpholinoethyl)-1*H*-1,2,3-triazol-4-yl)methoxy]phenyl}-1*H*-benzo[*d*]imidazole dihydrochloride (7a)

According to the above-mentioned procedure, from compound **3a** (150 mg, 0.47 mmol) compound **7a** was obtained as grey solid (74.5 mg, 33%, m.p. = 207–210 °C). ^{1}H NMR (300 MHz, DMSO-d_6) (δ/ppm): 11.98 (1H, bs, NH), 10.94 (2H, s, CNH), 8.50–8.40 (4H, m, H4; H5'; Ph) 8.04 (1H, dd, J = 8.6, 1.4 Hz, H6), 7.90 (1H, d, J = 8.6 Hz, H7), 7.33 (2H, d, J = 9.0 Hz, Ph), 5.33 (2H, s, OCH_2), 4.98 (2H, t, J = 7.0 Hz, CH_2CH_2), 4.02 (4H, s, $\underline{CH_2CH_2}$), 3.97–3.80 (4H, m, $CH_{2\text{-morpholine}}$), 3.69 (2H, t, J = 7.0 Hz, CH_2CH_2), 3.50–3.33 (2H, m, $CH_{2\text{- morpholine}}$), 3.22–3.03 (2H, m, $CH_{2\text{-morpholine}}$). ^{13}C NMR (75 MHz, DMSO-d_6) (δ/ppm): 164.9 (CNH), 161.3 (Ph-q), 153.0 (C2), 142.5 (C4'), 139.1 (Ph-q), 136.5 (C7a), 135.4 (C3a), 129.9 (Ph), 125.5 (C5'), 124.0 (C6), 117.1 (C5), 115.7 (C4), 115.6 (Ph), 114.7 (C7), 63.1 ($CH_{2\text{-morpholine}}$), 61.4 (OCH_2),

54.1 (CH_2CH_2), 51.2 ($CH_{2\text{-morpholine}}$), 44.3 (CH_2CH_2), 43.6 ($\underline{CH_2CH_2}$). Anal. calcd. for $C_{25}H_{28}N_8O_2 \times 2HCl \times 1.75H_2O$ (M_r = 576.99): C 52.04, H 5.85, N 19.42; found: C 52.27, H 5.97, N 19.26.

5-(Imidazolin-2-yl)-2-{4-[(1-(2-morpholinoethyl)-1*H*-1,2,3-triazol-4-yl)methoxy]-3-fluorophenyl}-1*H*-benzo[*d*]imidazole dihydrochloride (7b)

According to the above-mentioned procedure, from compound **3b** (150 mg, 0.45 mmol) compound **7b** was obtained as brown solid (169.6 mg, 72%, m.p. = 202–206 °C). ^{1}H NMR (300 MHz, DMSO-d_6) (δ/ppm): 14.17–13.55 (1H, m, NH), 10.66 (2H, s, CNH), 8.45–8.26 (2H, m, H5'; H4), 8.20–8.05 (2H, m, Ph; H6), 7.92–7.72 (2H, m, Ph; H7), 7.60 (1H, t, J = 8.6 Hz, Ph), 5.36 (2H, s, OCH_2), 4.52 (2H, t, J = 6.1 Hz, CH_2CH_2), 4.02 (4H, s, $\underline{CH_2CH_2}$), 3.60–3.41 (4H, m, $CH_{2\text{-morpholine}}$), 2.75 (2H, t, J = 5.5 Hz, CH_2CH_2), 2.41 (4H, bs, $CH_{2\text{-morpholine}}$). ^{13}C NMR (75 MHz, DMSO-d_6) (δ/ppm): 165.4 (CNH), 153.3 (C2), 153.2; 150.1 (d, J_{CF} = 244.1 Hz, Ph-q), 148.0; 147.9 (d, J_{CF} = 6.1 Hz, Ph-q), 141.6 (C4'), 125.4 (C5'), 123.9 (Ph), 122.5; 122.4 (d, J_{CF} = 7.2 Hz, Ph-q), 115.8 (Ph), 115.7 (C4), 115.3 (C5), 114.7; 114.5 (d, J_{CF} = 17.3 Hz, Ph), 112.2 (C7), 66.0 ($CH_{2\text{-morpholine}}$), 62.2 (OCH_2), 57.2 (CH_2CH_2), 52.9 ($CH_{2\text{-morpholine}}$), 46.5 (CH_2CH_2), 44.3 ($\underline{CH_2CH_2}$). Anal. calcd. for $C_{25}H_{27}FN_8O_2 \times 2HCl \times 2.25H_2O$ (M_r = 603.99): C 49.71, H 5.59, N 18.85; found: C 49.48, H 5.37, N 18.96.

5-(Imidazolin-2-yl)-2-{4-[(1-(2-morpholinoethyl)-1*H*-1,2,3-triazol-4-yl)methoxy]-3-methoxyphenyl}-1*H*-benzo[*d*]imidazole dihydrochloride (7c)

According to the above-mentioned procedure, from compound **3c** (150 mg; 0.43 mmol) compound **7c** was obtained as grey solid (184.1 mg, 85%, m.p. = 180–184 °C). ^{1}H NMR (600 MHz, DMSO-d_6) (δ/ppm): 13.94–13.44 (1H, m, NH), 10.60 (2H, m, CNH), 8.42–8.18 (2H, m, H5', H4), 7.98–7.70 (4H, m, Ph; H6; H7), 7.36 (1H, d, J = 8.4 Hz, Ph), 5.24 (2H, s, OCH_2), 4.52 (2H, t, J = 5.6 Hz, CH_2CH_2), 4.02 (4H, s, $\underline{CH_2CH_2}$), 3.89 (3H, s, OCH_3), 3.53 (4H, s, $CH_{2\text{-morpholine}}$), 2.75 (2H, s, CH_2CH_2), 2.42 (4H, s, $CH_{2\text{-morpholine}}$). ^{13}C NMR (151 MHz, DMSO-d_6) (δ/ppm): 164.8 (CNH), 153.0 (Ph-q), 150.9 (C5), 149.2 (Ph-q), 142.4 (C4'), 125.5 (Ph), 124.1 (C6), 121.6 (C5'), 117.2 (C5), 115.6 (C4), 113.5 (Ph, C7), 111.4 (Ph), 63.0 ($CH_{2\text{-morpholine}}$), 61.7 (OCH_2), 56.1 (OCH_3), 54.1 (CH_2CH_2), 51.2 ($CH_{2\text{-morpholine}}$), 44.3 (CH_2CH_2), 43.6 ($\underline{CH_2CH_2}$). Anal. calcd. for $C_{26}H_{30}N_8O_3 \times 2HCl \times H_2O$ (M_r = 593.51): C 52.62, H 5.77, N 18.88; found: C 52.29.48, H 5.94, N 18.73.

*4.2.6. General procedure for the synthesis of benzaldehydes **9a–13a**, **9b–13b** and **9c–13c***

The corresponding Hydroxybenzaldehyde (**1a**–**1c**) was dissolved in dry acetonitrile (15-20 mL) and K_2CO_3 (1.5 eq) was added. The reaction mixture was stirred for 30 min and the appropriate halide (1 eq) was added and stirring was continued overnight at reflux temperature. The course of the reaction was monitored by TLC. After completion of the reaction, the solvent was evaporated under reduced pressure and the residue was purified by column chromatography.

4-(2-Morpholino-2-oxoethoxy)benzaldehyde (9a)

Compound **9a** was prepared according to the general procedure from 4-hydroxybenzaldehyde **1a** (250 mg, 2.05 mmol) and *N*-(chloroacetyl)morpholine (0.27 mL, 2.05 mmol). After purification with column cromatography (CH_2Cl_2 : CH_3OH = 100 : 1) compound **9a** was obtained as white solid (506.7 mg, 99%, m.p. = 147–150 °C). ^{1}H NMR (300 MHz, DMSO-d_6) (δ/ppm): 9.87 (1H, s, CHO), 7.85 (2H, d, J = 8.8 Hz, Ph), 7.11 (2H, d, J = 8.7 Hz, Ph), 5.01 (2H, s, OCH_2), 3.68–3.52 (4H, m, $CH_{2\text{-morpholine}}$), 3.51–3.40 (4H, m, $CH_{2\text{-morpholine}}$). ^{13}C NMR (75 MHz, DMSO-d_6) (δ/ppm): 191.3 (CHO), 165.4 (CO), 163.1 (Ph-q), 131.6 (Ph), 129.8 (Ph-q), 115.1 (Ph), 66.0 ($CH_{2\text{-morpholine}}$), 65.6 (OCH_2), 44.6 ($CH_{2\text{-morpholine}}$), 41.6 ($CH_{2\text{-morpholine}}$).

3-Fluoro-4-(2-morpholino-2-oxoethoxy)benzaldehyde (9b)

Compound **9b** was prepared according to the general procedure from 3-fluoro-4-hydroxybenzaldehyde **1b** (250 mg, 1.78 mmol) and *N*-(chloroacetyl)morpholine (0.23 mL, 1.78 mmol). After purification with column cromatography (CH_2Cl_2 : CH_3OH = 50 : 1) compound **9b** was obtained as white solid (473.9 mg, 99%, m.p. = 84–88 °C). ^{1}H NMR (300 MHz, DMSO-d_6) (δ/ppm): 9.86 (1H, d, J = 2.0 Hz, CHO), 7.80–7.67 (2H, m, Ph), 7.28 (1H, t, J = 8.5 Hz, Ph), 5.13 (2H, s, OCH_2), 3.64–3.54 (4H, m, $CH_{2\text{-morpholine}}$), 3.47–3.42 (4H, m, $CH_{2\text{-morpholine}}$). ^{13}C NMR (75 MHz, DMSO-d_6) (δ/ppm): 190.8; 190.8 (d, J_{CF} = 1.9 Hz, CHO), 165.0 (CO), 153.2; 149.9 (d, J_{CF} = 247.0 Hz, Ph-q) 151.4; 151.3 (d, J_{CF} = 10.5 Hz, Ph-q), 129.9; 129.8 (d, J_{CF} = 5.2 Hz, Ph-q), 127.8; 127.8 (d, J_{CF} = 2.9 Hz, Ph), 115.5; 115.3 (d, J_{CF} = 18.3 Hz, Ph), 115.0; 115.0 (d, J_{CF} = 1.5 Hz), 66.1 ($CH_{2\text{-morpholine}}$), 65.9 (OCH_2), 44.5 ($CH_{2\text{-morpholine}}$), 41.6 ($CH_{2\text{-morpholine}}$).

3-Methoxy-4-(2-morpholino-2-oxoethoxy)benzaldehyde (9c)

Compound **9c** was prepared according to the general procedure from 3-methoxy-4-hydroxybenzaldehyde **1c** (250 mg, 1.64 mmol) and *N*-(chloroacetyl)morpholine (0.21 mL, 1.64 mmol). After purification with column cromatography (CH_2Cl_2 : CH_3OH = 50 : 1) compound

9c was obtained as white solid (452.3 mg, 99%, m.p. = 80–85 °C). ^{1}H NMR (300 MHz, DMSO-d_6) (δ/ppm): 9.84 (1H, s, CHO), 7.51 (1H, dd, J = 8.3, 1.9 Hz, Ph), 7.42 (1H, d, J = 1.8 Hz, Ph), 7.06 (1H, d, J = 8.3 Hz, Ph), 5.0 (2H, s, OCH_2), 3.85 (3H, s, OCH_3), 3.71–3.52 (4H, m, $CH_{2\text{-morpholine}}$), 3.50–3.39 (4H, m, $CH_{2\text{-morpholine}}$). ^{13}C NMR (75 MHz, DMSO-d_6) (δ/ppm): 191.4 (CHO), 165.3 (CO), 152.9 (Ph-q), 149.2 (Ph-q), 129.9 (Ph-q), 125.5 (Ph), 112.7 (Ph), 110.0 (Ph), 66.1 (OCH_2, $CH_{2\text{-morpholine}}$), 55.6 (OCH_3), 44.7 ($CH_{2\text{-morpholine}}$), 41.6 ($CH_{2\text{-morpholine}}$).

4-(2-Oxo-2-phenylethoxy)benzaldehyde (10a)

Compound **10a** was prepared according to the general procedure from 4-hydroxybenzaldehyde **1a** (250 mg, 2.5 mmol) and 2-bromoacetophenone (352.4 mg, 1.78 mmol). After purification with column cromatography (CH_2Cl_2 : CH_3OH = 50 : 1) compound **10a** was obtained as yellow solid (324.7 mg, 71%, m.p. = 81–85 °C). ^{1}H NMR (300 MHz, DMSO-d_6) (δ/ppm): 9.87 (1H, s, CHO), 8.04 (2H, d, J = 7.1 Hz, Ph), 7.86 (2H, d, J = 8.8 Hz, Ph), 7.71 (1H, t, J = 7.4 Hz, Ph), 7.58 (2H, t, J = 7.5 Hz, Ph), 7.17 (2H, d, J = 8.7 Hz, Ph), 5.77 (2H, s, OCH_2). ^{13}C NMR (151 MHz, DMSO-d_6) (δ/ppm): 193.7 (CO), 191.2 (CHO), 162.9 (Ph-q), 134.2 (Ph-q), 133.8 (Ph), 131.6 (Ph), 129.9 (Ph-q), 128.8 (Ph), 127.8 (Ph), 115.1 (Ph), 70.3 (OCH_2).

3-Fluoro-4-(2-oxo-2-phenylethoxy)benzaldehyde (10b)

Compound **10b** was prepared according to the general procedure from 3-fluoro-4-hydroxybenzaldehyde **1b** (250 mg, 1.78 mmol) and 2-bromoacetophenone (322.4 mg, 1.78 mmol). After purification with column cromatography (CH_2Cl_2 : CH_3OH = 50 : 1) compound **10b** was obtained as yellow solid (365.9 mg, 72%, m.p. = 115–120 °C). ^{1}H NMR (300 MHz, DMSO-d_6) (δ/ppm): 9.86 (1H, d, J = 2.0 Hz, CHO), 8.06–7.89 (2H, m, Ph), 7.83–7.65 (3H, m, Ph), 7.59 (2H, t, J = 7.6 Hz, Ph), 7.35 (1H, t, J = 8.5 Hz, Ph), 5.89 (2H, s, OCH_2). ^{13}C NMR (75 MHz, DMSO-d_6) (δ/ppm): 193.4 (CO), 190.8; 190.8 (d, J_{CF} = 1.8 Hz, CHO), 153.2; 149.9 (d, J_{CF} = 247.1 Hz, Ph-q), 151.3; 151.1 (d, J_{CF} = 10.6 Hz, Ph-q), 134.0 (Ph), 130.0; 129.9 (d, J_{CF} = 5.0 Hz, Ph-q), 128.9 (Ph), 128.0 (Ph), 127.9 (Ph), 115.6 (Ph), 115.4; 115.1 (d, J_{CF} = 18.3 Hz, Ph), 115.0 (Ph), 70.9 (OCH_2).

3-Methoxy-4-(2-oxo-2-phenylethoxy)benzaldehyde (10c)

Compound **10c** was prepared according to the general procedure from 3-methoxy-4-hydroxybenzaldehyde **1c** (250 mg, 1.64 mmol) and 2-bromoacetophenone (324.7 mg, 1.64 mmol). After purification with column cromatography (CH_2Cl_2 : CH_3OH = 50 : 1) compound

10c was obtained as white solid (424.7 mg, 96%, m.p. = 132–134 °C). ^{1}H NMR (300 MHz, DMSO-d_6) (δ/ppm): 9.84 (1H, s, CHO), 8.03 (2H, d, J = 7.2 Hz, Ph), 7.70 (1H, d, J = 7.4 Hz, Ph), 7.58 (2H, t, J = 7.5 Hz, Ph), 7.52–7.40 (2H, m, Ph), 7.10 (1H, d, J = 8.3 Hz, Ph), 5.77 (2H, s, OCH_2), 3.88 (3H, s, OCH_3). ^{13}C NMR (151 MHz, DMSO-d_6) (δ/ppm): 193.7 (CO), 191.3 (CHO), 152.8 (Ph-q), 149.2 (Ph-q), 134.2 (Ph-q), 133.9 (Ph), 130.0 (Ph-q), 128.8 (Ph), 127.9 (Ph), 125.5 (Ph), 112.7 (Ph), 110.1 (Ph), 70.5 (OCH_2), 55.6 (OCH_3).

4-(Piridin-2-yl-methoxy)benzaldehyde (11a)

Compound **11a** was prepared according to the general procedure from 4-hydroxybenzaldehyde **1a** (250 mg, 2.5 mmol) and 2-(chloromethyl)pyridine (403.5 mg, 2.05 mmol). After purification with column cromatography (CH_2Cl_2 : CH_3OH = 100 : 1) compound **11a** was obtained as white solid (385.3 mg, 88%, m.p. = 94–97 °C). ^{1}H NMR (300 MHz, DMSO-d_6) (δ/ppm): 9.87 (1H, s, CHO), 8.59 (1H, d, J = 4.2 Hz, H6'), 7.92–7.80 (3H, m, Ph, H4'), 7.53 (1H, d, J = 7.8 Hz, H3'), 7.36 (1H, dd, J = 6.8, 5.0 Hz, H5'), 7.23 (2H, d, J = 8.7 Hz, Ph), 5.31 (2H, s, OCH_2). ^{13}C NMR (75 MHz, DMSO-d_6) (δ/ppm): 191.3 (CHO), 163.1 (Ph-q), 155.9 (C2'), 149.2 (C6'), 137.1 (Ph), 131.8 (Ph-q), 129.9 (C4'), 123.2 (C3'), 121.9 (C5'), 115.3 (Ph), 70.6 (OCH_2).

3-Fluoro-4-(piridin-2-yl-methoxy)benzaldehyde (11b)

Compound **11b** was prepared according to the general procedure from 3-fluoro-4-hydroxybenzaldehyde **1b** (250 mg, 1.78 mmol) and 2-(chloromethyl)pyridine (351.0 mg, 1.78 mmol). After purification with column cromatography (CH_2Cl_2 : CH_3OH = 100 : 1) compound **11b** was obtained as white solid (307.6 mg, 75%, m.p. = 82–85 °C). ^{1}H NMR (300 MHz, DMSO-d_6) (δ/ppm): 9.87 (1H, d, J = 2.1 Hz, CHO), 8.76–8.33 (1H, m, 6'), 7.87 (1H, td, J = 7.7, 1.8 Hz, Ph), 7.80–7.70 (2H, m, Ph, H4'), 7.55 (1H, d, J = 7.8 Hz, H3'), 7.47 (1H, t, J = 8.2 Hz, Ph), 7.41–7.32 (1H, m, H5'), 5.39 (2H, s, OCH_2). ^{13}C NMR (151 MHz, DMSO-d_6) (δ/ppm):190.8 (CHO), 155.3 (Ph-q, C2') 152.5; 150.8 (d, J_{CF} = 246.9 Hz, Ph-q), 151.3; 151.2 (d, J_{CF} = 10.5 Hz, Ph-q), 149.2 (C6'), 137.1 (Ph), 130.0; 129.9 (d, J_{CF} = 5.1 Hz, Ph-q), 128.2 (C4'), 123.3 (C5'), 121.9 (C5'), 115.4; 115.3 (d, J_{CF} = 18.2 Hz, Ph), 115.1 (Ph), 71.3 (OCH_2).

3-Methoxy-4-(piridin-2-yl-methoxy)benzaldehyde (11c)

Compound **11c** was prepared according to the general procedure from 3-methoxy-4-hydroxybenzaldehyde **1c** (250 mg, 1.64 mmol) and 2-(chloromethyl)pyridine (323.14 mg, 1.64 mmol). After purification with column cromatography (CH_2Cl_2 : CH_3OH = 100 : 1) compound

11c was obtained as white solid (398.67 mg, 60%, m.p. = 77–81 °C). ^{1}H NMR (300 MHz, DMSO-d_6) (δ/ppm): 9.85 (1H, s, CHO), 8.59 (1H, d, J = 4.2 Hz, H6'), 7.86 (1H, td, J = 7.7, 1.7 Hz, Ph), 7.58–7.48 (2H, m, H4', H3'), 7.44 (1H, d, J = 1.8 Hz, Ph), 7.37 (1H, dd, J = 6.8, 5.1 Hz, H5'), 7.26 (1H, d, J = 8.3 Hz, Ph), 5.29 (2H, s, OCH_2), 3.87 (3H, s, OCH_3). ^{13}C NMR (151 MHz, DMSO-d_6) (δ/ppm): 191.4 (CHO), 155.9 (Ph-q), 152.9 (Ph-q), 149.4 (C2'), 149.2 (C6'), 137.1 (Ph), 130.0 (Ph-q), 125.8 (C4'), 123.2 (C3'), 121.9 (C5'), 112.7 (Ph), 109.9 (Ph), 70.9 (OCH_2), 55.6 (OCH_3).

4-(2-Morpholinoethoxy)benzaldehyde (12a)

Compound **12a** was prepared according to the general procedure from 4-hydroxybenzaldehyde **1a** (250 mg, 2.5 mmol) and 4-(2-chloroethyl)morpholine hydrochloride (340 mg, 2.05 mmol). After purification with column cromatography (CH_2Cl_2 : CH_3OH = 50 : 1) compound **12a** was obtained as colourless oil (425.2 mg, 88%). ^{1}H NMR (300 MHz, DMSO-d_6) (δ/ppm): 9.86 (1H, s, CHO), 7.85 (2H, d, J = 8.8 Hz, Ph), 7.13 (2H, d, J = 8.7 Hz, Ph), 4.20 (2H, t, J = 5.7 Hz, CH_2CH_2), 3.70–3.47 (4H, m, $CH_{2\text{-morpholine}}$), 2.71 (2H, t, J = 5.7 Hz, CH_2CH_2), 2.49–2.43 (4H, m, $CH_{2\text{-morpholine}}$). ^{13}C NMR (151 MHz, DMSO-d_6) (δ/ppm): 191.3 (CHO), 163.4 (Ph-q), 131.2 (Ph), 129.6 (Ph-q), 115.0 (Ph), 66.1 ($CH_{2\text{-morpholine}}$), 65.8 (CH_2CH_2), 56.8 (CH_2CH_2), 53.5 ($CH_{2\text{-morpholine}}$).

3-Fluoro-4-(2-morpholinoethoxy)benzaldehyde (12b)

Compound **12b** was prepared according to the general procedure from 3-fluoro-4-hydroxybenzaldehyde **1b** (250 mg, 1.78 mmol) and 4-(2-chloroethyl)morpholine hydrochloride (331.22 mg, 1.78 mmol). After purification with column cromatography (CH_2Cl_2 : CH_3OH = 50 : 1) compound **12b** was obtained as yellow oil (430.4 mg, 95%). ^{1}H NMR (300 MHz, DMSO-d_6) (δ/ppm): 9.86 (1H, d, J = 2.1 Hz, CHO), 7.79–7.74 (1H, m, Ph), 7.68 (1H, dd, J = 11.4, 1.9 Hz, Ph), 7.41 (1H, t, J = 8.3 Hz, Ph), 4.29 (2H, t, J = 5.7 Hz, CH_2CH_2), 3.64–3.46 (4H, m, $CH_{2\text{-morpholine}}$), 2.74 (2H, t, J = 5.7 Hz, CH_2CH_2), 2.49–2.43 (4H, m, $CH_{2\text{-morpholine}}$). ^{13}C NMR (75 MHz, DMSO-d_6) (δ/ppm): 190.8 (CHO), 153.3; 150.0 (d, J_{CF} = 246.9 Hz, Ph-q), 151.8; 151.6 (d, J_{CF} = 10.7 Hz, Ph-q), 129.7; 129.6 (d, J_{CF} = 4.9 Hz, Ph-q), 128.3; 128.3 (d, J_{CF} = 3.1 Hz, Ph), 115.3; 115.1 (d, J_{CF} = 18.3 Hz, Ph), 114.7; 114.7 (d, J_{CF} = 1.6 Hz, Ph), 67.0 ($CH_{2\text{-morpholine}}$), 66.1 (CH_2CH_2), 56.6 (CH_2CH_2), 53.5 ($CH_{2\text{-morpholine}}$).

3-Methoxy-4-(2-morpholinoethoxy)benzaldehyde (12c)

Compound **12c** was prepared according to the general procedure from 3-methoxy-4-hydroxybenzaldehyde **1c** (250 mg, 1.64 mmol) and 4-(2-chloroethyl)morpholine hydrochloride (305.17 mg, 1.64 mmol). After purification with column cromatography (CH_2Cl_2 : CH_3OH = 50 : 1) compound **12c** was obtained as yellow oil (415.9 mg, 96%). ^{1}H NMR (300 MHz, DMSO-d_6) (δ/ppm): 9.84 (1H, s, CHO), 7.53 (1H, dd, J = 8.2, 1.9 Hz, Ph), 7.39 (1H, d, J = 1.8 Hz, Ph), 7.20 (1H, d, J = 8.3 Hz, Ph), 4.19 (2H, t, J = 5.9 Hz, CH_2CH_2), 3.83 (3H, s, OCH_3), 3.62–3.45 (4H, m, $CH_{2\text{-morpholine}}$), 2.72 (2H, t, J = 5.8 Hz, CH_2CH_2), 2.49–2.43 (4H, m, $CH_{2\text{-morpholine}}$). ^{13}C NMR (75 MHz, DMSO-d_6) (δ/ppm): 191.4 (CHO), 153.4 (Ph-q), 149.3 (Ph-q), 129.7 (Ph-q), 126.0 (Ph), 112.3 (Ph), 109.7 (Ph), 66.4 (CH_2CH_2), 66.2 ($CH_{2\text{-morpholine}}$), 56.8 (CH_2CH_2), 55.6 (OCH_3), 53.6 ($CH_{2\text{-morpholine}}$).

4-(2-(Diethylamino)ethoxy)benzaldehyde (13a)

Compound **13a** was prepared according to the general procedure from 4-hydroxybenzaldehyde **1a** (250 mg; 2,5 mmol) and 2-chloro-*N*,*N*-dimethylethylamine hydrochloride (339.42 mg, 2.05 mmol). After purification with column cromatography (CH_2Cl_2 : CH_3OH = 50 : 1) compound **13a** was obtained as yellow oil (136.4 mg, 30%). ^{1}H NMR (300 MHz, DMSO-d_6) (δ/ppm): 9.86 (1H, s, CHO), 7.85 (2H, d, J = 8.8 Hz, Ph), 7.12 (2H, d, J = 8.7 Hz, Ph), 4.14 (2H, t, J = 6.0 Hz, CH_2CH_2), 2.81 (2H, t, J = 5.9 Hz, CH_2CH_2) 2.62–2.51 (4H, m, $\underline{CH_2}CH_3$), 0.97 (6H, t, J = 7.1 Hz, $CH_2\underline{CH_3}$). ^{13}C NMR (75 MHz, DMSO-d_6) (δ/ppm): 191.8 (CHO), 164.0 (Ph-q), 132.3 (Ph), 130.0 (Ph-q), 115.4 (Ph), 67.3 (CH_2CH_2), 51.6 (CH_2CH_2), 47.4 ($\underline{CH_2}CH_3$), 12.2 ($CH_2\underline{CH_3}$).

4-(2-(Diethiylamino)ethoxy)-3-fluorobenzaldehyde (13b)

Compound **13b** was prepared according to the general procedure from 3-fluoro-4-hydroxybenzaldehyde **1b** (250 mg, 1.78 mmol) and 2-chloro-*N*,*N*-dimethylethylamine hydrochloride (295.87 mg, 1.78 mmol). After purification with column cromatography (CH_2Cl_2 : CH_3OH = 50 : 1) compound **13b** was obtained as yellow oil (335.1 mg, 79%). ^{1}H NMR (300 MHz, DMSO-d_6) (δ/ppm): 9.82 (1H, d, J = 2.1 Hz, CHO), 7.72 (1H, d, J = 8.5 Hz, Ph), 7.64 (1H, dd, J = 11.4, 1.9 Hz, Ph), 7.37 (1H, t, J = 8.3 Hz, Ph), 4.17 (2H, t, J = 5.9 Hz, CH_2CH_2), 2.92–2.70 (2H, m, CH_2CH_2), 2.48–2.41 (4H, m, $\underline{CH_2}CH_3$), 0.93 (6H, t, J = 7.1 Hz, $CH_2\underline{CH_3}$). ^{13}C NMR (151 MHz, DMSO-d_6) (δ/ppm): 190.7 (CHO), 152.5; 150.8 (d, J_{CF} = 247.0 Hz, Ph-q), 151.8; 151.7 (d, J_{CF} = 10.7 Hz, Ph-q), 129.6; 129.5 (d, J_{CF} = 5.1 Hz, Ph-q), 128.2; 128.2 (d,

J_{CF} = 2.7 Hz, Ph), 116.1; 116.0 (, J_{CF} = 18.3 Hz, Ph), 114.6 (Ph), 67.8 (CH_2CH_2), 50.9 (CH_2CH_2),), 47.0 ($\underline{CH_2}CH_3$), 11.7 ($CH_2\underline{CH_3}$).

4-(2-(Dietihylamino)ethoxy)-3-methoxybenzaldehyde (13c)

Compound **13c** was prepared according to the general procedure from 3-methoxy-4-hydroxybenzaldehyde **1c** (250 mg; 1.64 mmol) and 2-chloro-*N*,*N*-dimethylethylamine hydrochloride (271.98 mg, 1.64 mmol). After purification with column cromatography (CH_2Cl_2 : CH_3OH = 50 : 1) compound **13c** was obtained as yellow oil (354.2 mg, 66%). ^{1}H NMR (300 MHz, DMSO-d_6) (δ/ppm): 9.83 (1H, s, CHO), 7.53 (1H, dd, J = 8.2, 1.9 Hz, Ph), 7.39 (1H, d, J = 1.8 Hz, Ph), 7.19 (1H, d, J = 8.3 Hz, Ph), 4.12 (2H, t, J = 6.2 Hz, CH_2CH_2), 3.82 (3H, s, OCH_3), 2.81 (2H, t, J = 6.2 Hz, CH_2CH_2), 2.66–2.50 (4H, m, $\underline{CH_2}CH_3$), 0.97 (6H, t, J = 7.1 Hz, $CH_2\underline{CH_3}$). ^{13}C NMR (75 MHz, DMSO-d_6) (δ/ppm): 191.4 (CHO), 153.5 (Ph-q), 149.3 (Ph-q), 129.6 (Ph-q), 126.1 (Ph), 112.2 (Ph), 109.7 (Ph), 67.3 (CH_2CH_2), 55.6 (OCH_3), 51.1 (CH_2CH_2), 47.0 ($\underline{CH_2}CH_3$), 11.8 ($CH_2\underline{CH_3}$).

*4.2.7. General procedure for the synthesis of benzimidazole derivaties **14a–18a**, **14b–18b** and **14c–18c***

The reaction mixture of the corresponding benzaldehyde derivative (**9a**–**13a**, **9b**–**13b**, **9c**–**13c**), 4-(imidazolin-2-yl)benzene-1,2-diamine (1 eq) and 40% $NaHSO_3$ (aq) was dissolved in 15 ml EtOH and stirred under reflux for 6–8 h. After completition of the reaction $NaHSO_3$ was filtered and the reaction mixture was evaporated to dryness. The crude residue was purified by column chromatography (CH_2Cl_2 : CH_3OH = 4 : 1). The obtained solid was dissolved in HCl saturated EtOH (8–10 mL) and stirred for 4 h. Addition of ether resulted in precipitation of products **14a**–**18a**, **14b**–**18b** and **14c**–**18c**. Solid was collected by filtration, washed with anhydrous ether, and dried under vacuum.

5-(Imidazolin-2-yl)-2-[4-(2-morpholino-2-oxoethoxy)phenyl]-1*H*-benzo[*d*]imidazole dihydrochloride (14a)

According to the above-mentioned procedure, from **9a** (200 mg, 0.80 mmol) compound **14a** was obtained as brown solid (118.6 mg, 37%, m.p. = 220–223 °C). ^{1}H NMR (300 MHz, DMSO-d_6) (δ/ppm): 10.82 (2H, s, CNH), 8.45 (1H, s, H4), 8.39 (2H, d, J = 8.5 Hz, Ph), 8.00 (1H, d, J = 8.3 Hz, H7), 7.89 (1H, d, J = 8.5 Hz, H6), 7.20 (2H, d, J = 8.9 Hz, Ph), 5.01 (2H, s, CH_2), 4.03 (4H, s, $\underline{CH_2}\underline{CH_2}$), 3.73–3.35 (8H, m, $CH_{2\text{-morpholine}}$). ^{13}C NMR (75 MHz, DMSO-d_6)

(δ/ppm): 165.6 (CNH), 165.1 (CO), 161.3 (Ph-q), 153.5 (C2), 129.5 (Ph), 123.7 (C6), 118.9 (C5), 116.8 (Ph-q), 115.9 (C4), 115.5 (Ph), 114.8 (C7), 66.1 ($CH_{2\text{-morpholine}}$), 65.7 (OCH_2), 44.4 ($\underline{CH_2}\underline{CH_2}$), 44.6 ($CH_{2\text{-morpholine}}$), 41.6 ($CH_{2\text{-morpholine}}$). Anal. calcd. for $C_{22}H_{23}N_5O_3 \times 2HCl \times 2.75H_2O$ (M_r = 527.91): C 50.05, H 5.82, N 13.27; found: C 50.28, H 5.94, N 13.11.

2-[3-Fluoro-4-(2-morpholino-2-oxoethoxy)phenyl]-5-(imidazolin-2-yl)-1*H*-benzo[*d*]imidazole dihydrochloride (14b)

According to the above-mentioned procedure, from **9b** (200 mg, 0.74 mmol) compound **14b** was obtained as brown solid (265.4 mg, 85%, m.p. = 211–215 °C). ^{1}H NMR (300 MHz, DMSO-d_6) (δ/ppm): 13.87 (1H, s, NH), 10.67 (2H, s, CNH), 8.36 (1H, s, H4), 8.17–8.01 (2H, m, H6, Ph), 7.91–7.72 (2H, m, H7, Ph), 7.29 (1H, t, J = 8.7 Hz, Ph), 5.09 (2H, s, OCH_2), 4.01 (4H, s, $\underline{CH_2}\underline{CH_2}$), 3.69–3.40 (8H, m, $CH_{2\text{-morpholine}}$). ^{13}C NMR (151 MHz, DMSO-d_6) (δ/ppm): 165.4 (CNH), 165.3 (CO), 152.2; 150.6 (d, J_{CF} = 244.3 Hz, Ph-q), 149.7 (Ph-q), 148.1; 148.1 (d, J_{CF} = 10.2 Hz, Ph-q), 123.7 (Ph, C6), 122.4; 122.3 (d, J_{CF} = 7.1 Hz, Ph-q), 115.6 (C4, Ph), 115.5 (C5), 115.3 (C7), 114.6; 114.5 (d, J_{CF} = 20.9 Hz, Ph), 66.1 ($CH_{2\text{-morpholine}}$), 65.9 (OCH_2), 44.6 ($CH_{2\text{-morpholine}}$), 44.2 ($\underline{CH_2}\underline{CH_2}$), 41.6 ($CH_{2\text{-morpholine}}$). Anal. calcd. for $C_{22}H_{22}FN_5O_3 \times 2HCl \times 3H_2O$ (M_r = 550.41): C 48.01, H 5.49, N 12.72; found: C 47.79, H 5.31, N 13.02.

5-(Imidazolin-2-yl)-2-[3-methoxy-4-(2-morpholino-2-oxoethoxy)phenyl]-1*H*-benzo[*d*]imidazole dihydrochloride (14c)

According to the above-mentioned procedure, from **9c** (200 mg, 0.72 mmol) compound **14c** was obtained as brown solid (269.7 mg, 86%, m.p. = 202–205 °C). ^{1}H NMR (300 MHz, DMSO-d_6) (δ/ppm): 13.78 (1H, bs, NH), 10.67 (2H, s, CNH), 8.36 (1H, s, H4), 8.00–7.70 (4H, m, Ph; H7; H6), 7.07 (1H, d, J = 8.6 Hz, Ph), 4.94 (2H, s, OCH_2), 4.01 (4H, s, $\underline{CH_2}\underline{CH_2}$), 3.92 (3H, s, OCH_3), 3.73–3.40 (8H, m, $CH_{2\text{-morpholine}}$). ^{13}C NMR (75 MHz, DMSO-d_6) (δ/ppm): 165.7 (CNH), 165.4 (Ph-q), 149.7 (C2), 149.0 (Ph-q), 122.3 (C6), 122.2 (Ph-q), 119.9 (Ph), 115.6 (C4, C7), 115.0 (Ph-q), 113.4 (Ph), 110.7 (Ph), 66.3 ($CH_{2\text{-morpholine}}$), 66.0 (OCH_2), 55.9 (OCH_3), 44.8 ($\underline{CH_2}\underline{CH_2}$), 44.2 ($CH_{2\text{-morpholine}}$), 41.6 ($CH_{2\text{-morpholine}}$). Anal. calcd. for $C_{23}H_{25}N_5O_4 \times 2HCl \times 2.25H_2O$ (M_r = 548.93): C 50.32, H 5.78, N 12.76; found: C 50.47, H 5.43, N 13.11.

5-(Imidazolin-2-yl)-2-[4-(2-oxoethoxy-2-phenyl)phenyl]-1*H*-benzo[*d*]imidazole hydrochloride (15a)

According to the above-mentioned procedure, from **10a** (150 mg, 0.62 mmol) compound **15a** was obtained as brown solid (205.0 mg, 83%, m.p. = 255–258 °C). ^{1}H NMR (300 MHz, DMSO-d_6) (δ/ppm): 13.67 (1H, s, NH), 10.62 (2H, s, CNH), 8.33 (1H, s, H4), 8.22 (2H, d, J = 8.6 Hz, Ph), 8.06 (2H, d, J = 7.2 Hz, Ph), 7.88–7.65 (3H, m, H6; H7; Ph), 7.59 (2H, t, J = 7.5 Hz, Ph), 7.19 (2H, d, J = 8.9 Hz, Ph), 5.73 (2H, s, OCH_2), 4.01 (4H, s, $\underline{CH_2CH_2}$). ^{13}C NMR (75 MHz, DMSO-d_6) (δ/ppm): 194.2 (CO), 165.4 (CNH), 160.1 (C2), 134.3 (Ph-q), 133.9 (Ph), 128.9 (Ph), 128.7 (Ph), 127.9 (Ph), 122.2 (C6), 122.0 (C5), 115.2 (C4, C7), 115.0 (Ph-q), 70.3 (OCH_2), 44.2 ($\underline{CH_2CH_2}$). Anal. calcd. for $C_{24}H_{20}N_4O_2 \times HCl \times 2H_2O$ (M_r = 468.93): C 61.47, H 5.37, N 11.95; found: C 61.09, H 4.98, N 12.30.

2-[3-Fluoro-4-(2-oxoethoxy-2-phenyl)phenyl]-5-(imidazolin-2-yl)-1*H*-benzo[*d*]imidazole hydrochloride (15b)

According to the above-mentioned procedure, from **10b** (150 mg, 0.66 mmol) compound **15b** was obtained as yellow solid (187.2 mg, 68%, m.p. = 205–208 °C). ^{1}H NMR (300 MHz, DMSO-d_6) (δ/ppm): 10.91 (2H, s, CNH), 8.48 (1H, s, H4), 8.39 (1H, dd, J = 12.3, 1.9 Hz, Ph), 8.25 (1H, d, J = 8.8 Hz, H7), 8.09–7.97 (3H, m, Ph;H6), 7.91 (1H, d, J = 8.6 Hz, Ph), 7.72 (1H, t, J = 7.4 Hz, Ph), 7.60 (2H, t, J = 7.5 Hz, Ph), 7.47 (1H, t, J = 8.8 Hz, Ph), 5.90 (2H, s, OCH_2), 4.02 (4H, s, $\underline{CH_2CH_2}$). ^{13}C NMR (75 MHz, DMSO-d_6) (δ/ppm): 193.5 (CO), 165.2 (CNH), 152.9 (C2), 152.9; 149.7 (d, J_{CF} = 238.2 Hz, Ph-q), 148.5; 148.3 (d, J_{CF} = 10.3 Hz, Ph-q), 134.1 (Ph-q), 133.6 (Ph), 128.6 (Ph), 127.6 (Ph), 124.1; 124.1 (d, J_{CF} = 1.3 Hz, Ph), 122.9 (C6), 120.9 (Ph-q), 116.3 (C4), 116.0 (C5), 115.6 (C7), 115.1; 114.8 (d, J_{CF} = 20.9 Hz, Ph), 114.8; 114.7 (d, J_{CF} = 5.1 Hz, Ph), 70.9 (OCH_2), 44.1 ($\underline{CH_2CH_2}$). Anal. calcd. for $C_{24}H_{19}FN_4O_2 \times HCl \times 1.5H_2O$ (M_r = 477.92): C 60.32, H 4.85, N 11.72; found: C 60.03, H 4.98, N 12.07.

2-[3-Fluoro-4-(2-oxoethoxy-2-phenyl)phenyl]-5-(imidazolin-2-yl)-1*H*-benzo[*d*]imidazole hydrochloride (15c)

According to the above-mentioned procedure, from **10c** (150 mg, 0.55 mmol) compound **15c** was obtained as brown solid (183.6 mg, 78%, m.p. = 206–210 °C). ^{1}H NMR (300 MHz, DMSO-d_6) (δ/ppm): 10.66 (2H, s, CNH), 8.34 (1H, s, H4), 8.05 (2H, d, J = 7.2 Hz, Ph), 7.97–7.67 (5H, m, Ph), 7.59 (2H, t, J = 7.5 Hz, Ph), 7.11 (1H, d, J = 8.6 Hz, Ph), 5.71 (2H, s, OCH_2), 4.01 (4H, s, $\underline{CH_2CH_2}$), 3.95 (3H, s, OCH_3). ^{13}C NMR (75 MHz, DMSO-d_6) (δ/ppm): 194.2 (CO), 165.5 (CNH), 153.7 (C2), 149.7 (Ph-q), 149.0 (Ph-q), 134.3 (Ph-q), 133.9 (Ph), 128.9 (Ph), 127.9 (Ph), 122.3 (Ph), 122.0 (Ph), 120.0 (C6), 115.1 (C5), 113.4 (Ph, C7), 110.7 (Ph), 70.6 (OCH_2),

55.9 (OCH_3), 44.2 ($\underline{CH_2}\underline{CH_2}$). Anal. calcd. for $C_{25}H_{22}N_4O_3 \times HCl \times 2.25H_2O$ (M_r = 503.46): C 59.64, H 5.51, N 11.13; found: C 60.06, H 5.21, N 11.37.

5-(Imidazolin-2-yl)-2-[4-(pyridin-2-ylmethoxy)phenyl]-1*H*-benzo[*d*]imidazole hydrochloride (16a)

According to the above-mentioned procedure, from **11a** (150 mg; 0.70 mmol) compound **16a** was obtained as white solid (175.0 mg, 46%, m.p. = 208–211 °C). ^{1}H NMR (600 MHz, DMSO-d_6) (δ/ppm): 11.03 (2H, s, CNH), 8.80 (1H, d, J = 4.9 Hz, H6'), 8.54-8.51 (3H, m, H4, Ph), 8.31-8.22 (1H, m, H4'), 8.14-8.07 (1H, m, H6), 7.92 (1H, d, J = 8.2 Hz, H7), 7.90 (1H, d, J = 7.8 Hz, H3'), 7.73 (1H, dd, J = 7.1, 5.1 Hz, H5'), 7.41 (2H, d, J = 8.8 Hz, Ph), 5.54 (2H, s, OCH_2), 4.03 (4H, s, $\underline{CH_2}\underline{CH_2}$). ^{13}C NMR (75 MHz, DMSO-d_6) (δ/ppm): 164.8 (CNH), 161.1 (Ph-q), 154.2 (C2'), 152.8 (C2), 146.8 (C6'), 139.7 (Ph), 138.9 (Ph), 135.3 (Ph-q), 129.9 (C4'), 124.0 (C3'), 123.0 (C5'), 118.53, 117.0 (C5), 115.6 (C4), 115.6 (Ph), 114.4 (C7), 69.0 (OCH_2), 44.2 ($\underline{CH_2}\underline{CH_2}$). Anal. calcd. for $C_{22}H_{19}N_5O \times HCl \times 2H_2O$ (M_r = 441.46): C 59.79, H 5.47, N 15.85; found: C 59.41, H 5.26, N 16.17.

5-(Imidazolin-2-yl)-2-[3-fluoro-4-(pyridin-2-ylmethoxy)phenyl]-1*H*-benzo[*d*]imidazole hydrochloride (l6b)

According to the above-mentioned procedure, from **11b** (150 mg, 0.65 mmol) compound **16b** was obtained as brown solid (187.2 mg, 74%, m.p. = 200–203 °C). ^{1}H NMR (300 MHz, DMSO-d_6) (δ/ppm): 10.91 (2H, s, CNH), 8.74 (1H, d, J = 4.5 Hz, H6'), 8.48 (1H, s, H4), 8.39 (1H, dd, J = 12.3, 1.9 Hz, H6), 8.28 (1H, d, J = 8.7 Hz, H7), 8.14 (1H, td, J = 7.8, 1.5 Hz, Ph), 8.01 (1H dd, J = 8.5, 1.5 Hz, H4'), 7.88 (1H, d, J = 8.6 Hz, H3'), 7.79 (1H, d, J = 7.9 Hz, Ph), 7.65–7.51 (2H, m, Ph; H5'), 5.52 (2H, s, OCH_2), 4.02 (4H, s, $\underline{CH_2}\underline{CH_2}$). ^{13}C NMR (75 MHz, DMSO-d_6) (δ/ppm): 165.1 (CNH), 154.9 (C2'), 154.1 (C2), 153.2; 149.9 (d, J_{CF} = 245.1 Hz, Ph-q),152.7 (C2), 148.7; 148.6 (d, J_{CF} = 10.5 Hz, Ph-q), 147.4 (C6'), 139.8 (Ph), 136.4 (Ph-q), 124.8; 124.8 (d, J_{CF} = 3.1 Hz, Ph), 124.6 (C4'), 124.3 (C3'), 123.5 (C6), 123.1 (C5'), 118.2 (C5), 116.8 (C4), 115.8 (C7), 115.5; 115.2 (d, J_{CF} = 21.2 Hz, Ph), 70.0 (OCH_2), 44.4 ($\underline{CH_2}\underline{CH_2}$).). Anal. calcd. for $C_{22}H_{18}FN_5O \times HCl \times 1.75H_2O$ (M_r = 455.40): C 58.02, H 4.98, N 15.38; found: C 57.63, H 5.26, N 15.57.

5-(Imidazolin-2-yl)-2-[3-methoxy-4-(pyridin-2-ylmethoxy)phenyl]-1*H*-benzo[*d*]imidazole hydrochloride (l6c)

According to the above-mentioned procedure, from **11c** (150 mg; 0,62 mmol) compound **16c** was obtained as brown solid (181.7 mg; 73%, m.p. = 265–268 °C). ^{1}H NMR (600 MHz, DMSO-d_6) (δ/ppm): 10.67 (2H, s, CNH), 8.60 (1H, d, J = 4.6 Hz, H6'), 8.35 (1H, s, H4), 7.96 (1H, s, Ph), 7.92–7.81 (3H, m, Ph; H4'; H6), 7.77 (1H, d, J = 8.2 Hz, H7), 7,56 (1H, d, J = 7.9 Hz, H3'), 7.37 (1H, dd, J = 7.2, 5.1 Hz, H5'), 7.24 (1H, d, J = 8.5 Hz, Ph), 5.27 (2H, s, OCH_2), 4.01 (4H, s, $\underline{CH_2CH_2}$), 3.94 (3H, s, OCH_3). ^{13}C NMR (75 MHz, DMSO-d_6) (δ/ppm): 165.4 (CNH), 156.4 (C2), 149.8 (Ph-q), 149.2 (Ph-q), 149.1 (C6'), 137.1 (Ph), 123.1 (C4'), 122.3 (C6), 122.2 (C5), 121.8 (C5'), 120.1 (C3'), 115.1 (Ph-q), 113.4 (C4/C7), 110.6 (Ph), 70.9 (OCH_2), 55.9 (OCH_3), 44.2 ($\underline{CH_2CH_2}$). Anal. calcd. for $C_{23}H_{21}N_5O_2 \times HCl \times 3H_2O$ (M_r = 489.95): C 56.38, H 5.76, N 14.29; found: C 56.03, H 5.39, N 14.57.

5-(Imidazolin-2-yl)-2-[4-(2-morpholinoethoxy)phenyl]1-1*H*-benzo[*d*]imidazole hydrochloride (l7a)

According to the above-mentioned procedure, from **12a** (150 mg; 0.64 mmol) compound **17a** was obtained as brown solid (248.5 mg, 74%, m.p. = 203–207 °C). ^{1}H NMR (600 MHz, DMSO-d_6) (δ/ppm): 11.82 (1H, s, NH), 10.96 (2H, s, CNH), 8.49–8.44 (3H, m, H4, Ph), 8.04 (1H, dd, J = 8.6, 1.3 Hz, H6), 7.89 (1H, d, J = f8.6 Hz, H7), 7.28 (2H, d, J = 8.9 Hz, Ph), 4.61 (2H, t, , J = 5.1 Hz, CH_2CH_2), 4.02 (4H, s, $\underline{CH_2CH_2}$), 3.96–3.87 (6H, m, $CH_{2\text{-morpholine}}$, CH_2CH_2), 3.64–3.40 (4H, m, $CH_{2\text{-morpholine}}$, CH_2CH_2). ^{13}C NMR (151 MHz, DMSO-d_6) (δ/ppm): 165.2 (CNH), 159.6 (Ph-q), 154.0 (C2), 129.0 (Ph), 122.6 (C6), 121.3 (Ph-q), 115.5 (C5), 115.2 (Ph), 115.0 (C4, C7), 62.9 ($CH_{2\text{-morpholine}}$), 62.4 (CH_2CH_2), 54.5 (CH_2CH_2), 51.5 ($CH_{2\text{-morpholine}}$), 44.0 ($\underline{CH_2CH_2}$).

5-(Imidazolin-2-yl)-2-[3-fluoro-4-(2-morpholinoethoxy)phenyl]1-1*H*-benzo[*d*]imidazole dihydrochloride (l7b)

According to the above-mentioned procedure, from **12b** (150 mg, 0.59 mmol) compound **17b** was obtained as brown solid (301.4 mg, 74%, m.p. = 204–208 °C). ^{1}H NMR (300 MHz, DMSO-d_6) (δ/ppm): 12.00 (1H, bs, NH), 10.99 (2H, s, CNH), 8.50 (1H, s, H4), 8.44–8.25 (2H, m, Ph), 8.04 (1H, dd, J = 8.6, 1.4 Hz, H6), 7.86 (1H, d, J = 8.6 Hz, H7), 7.53 (1H, t, J = 8.7 Hz, Ph), 4.75–4.68 (2H, m, CH_2CH_2), 4.01 (4H, s, $\underline{CH_2CH_2}$), 3.98–3.80 (4H, m, $CH_{2\text{- morpholine}}$), 3.70–3.61 (2H, m, $CH_{2\text{-morpholine}}$), 3.56–3.45 (2H, m, $CH_{2\text{-morpholine}}$) 3.33–3.18 (2H, m, CH_2CH_2). ^{13}C NMR (75 MHz, DMSO-d_6) (δ/ppm): 165.1 (CNH), 153.1; 149.8 (d, J_{CF} = 244.9 Hz, Ph-q), 152.8 (C2), 147.8; 147.5 (J_{CF} = 10.0 Hz, Ph-q), 124.3; 124.3 (J_{CF} = 1.5 Hz, Ph), 122.9 (C6),

121.9; 121.8 (J_{CF} = 7.5 Hz, Ph-q), 116.5 (C4), 115.8 (C5), 115.7 (Ph), 115.1; 114.8 (d, J_{CF} = 20.6 Hz, Ph), 114.7 (C7), 63.8 (CH_2CH_2), 62.9 ($CH_{2\text{-morpholine}}$), 54.4 (CH_2CH_2), 51.5 ($CH_{2\text{-morpholine}}$), 44.0 ($\underline{CH_2}CH_2$). Anal. calcd. for $C_{22}H_{24}FN_5O_2 \times 2HCl \times 2.5H_2O$ (M_r = 527.42): C 50.10, H 5.92, N 13.28; found: C 50.03, H 5.71, N 13.72.

5-(Imidazolin-2-yl)-2-[3-methoxy-4-(2-morpholinoethoxy)phenyl]1-1*H*-benzo[*d*]imidazole fdihydrochloride (l7c)

According to the above-mentioned procedure, from **12c** (150 mg; 0,56 mmol) compound **17c** was obtained as yellow solid (237.7 mg, 87%, m.p. = 217–223 °C). ^{1}H NMR (300 MHz, DMSO-d_6) (δ/ppm): ^{1}H NMR (300 MHz, DMSO) δ 12.08 (1H, bs, NH), 10.97 (2H, s, CNH), 8.50 (1H, s, H4), 8.24 (1H, d, J = 1.8 Hz, Ph), 8.15–8.01 (2H, m, Ph, H6), 7.87 (1H, d, J = 8.6 Hz, H7), 7.31 (1H, d, J = 8.6 Hz, Ph), 4.63 (2H, t, J = 5.1 Hz, CH_2CH_2), 4.02 3H, s, OCH_3), 3.97–3.88 (8H, m, $\underline{CH_2}\underline{CH_2}$, $CH_{2\text{-morpholine}}$), 3.65–3.40 (4H, m, $CH_{2\text{-morpholine}}$, CH_2CH_2), 3.32–3.20 (2H, m, $CH_{2\text{-morpholine}}$). ^{13}C NMR (151 MHz, DMSO-d_6) (δ/ppm): 165.0 (CNH), 153.3 (C2), 149.8 (Ph-q), 149.4 (Ph-q), 123.3 (Ph), 121.0 (C6), 120.5 (Ph-q), 116.2 (C5), 115.9 (C4), 114.4 (Ph, C7), 111.9 (Ph), 63.6 (CH_2CH_2), 62.8 ($CH_{2\text{-morpholine}}$), 56.2 (OCH_3), 54.5 (CH_2CH_2), 51.5 ($CH_{2\text{-morpholine}}$), 44.0 ($\underline{CH_2}CH_2$). Anal. calcd. for $C_{23}H_{27}N_5O_3 \times 2HCl \times 2H_2O$ (M_r = 530.44): C 52.08, H 6.27, N 13.20; found: C 52.43, H 5.98, N 13.11.

2-{4-[2-(Diethylamino)ethoxy]phenyl}-5-(imidazolin-2-yl)-1*H*-benzo[*d*]imidazole dihydrochloride (l8a)

According to the above-mentioned procedure, from **13a** (150 mg, 0.68 mmol) compound **18a** was obtained as brown solid (126.8 mg, 49%, m.p. = 214–218 °C). ^{1}H NMR (300 MHz, DMSO-d_6) (δ/ppm): 10.98 (3H, s, CNH), 8.54–8.40 (3H, m, Ph, H4), 8.06 (1H, dd, J = 8.6, 1.3 Hz, H6), 7.90 (1H, d, J = 8.6 Hz, H7), 7.28 (2H, d, J = 8.9 Hz, Ph), 4.56 (2H, t, J = 4.8 Hz, CH_2CH_2), 4.02 (4H, s, $\underline{CH_2}CH_2$), 3.63–3.44 (2H, m, CH_2CH_2), 3.29–3.10 (4H, m, $\underline{CH_2}CH_3$), 1.28 (6H, t, J = 7.2 Hz, $CH_2\underline{CH_3}$). ^{13}C NMR (75 MHz, DMSO-d_6) (δ/ppm): 164.9 (CNH), 160.6 (Ph-q), 153.2 (C2), 129.8 (Ph), 123.9 (C6), 120.5 (Ph-q), 116.8 (C5), 115.4 (Ph), 114.7 (C4, C7), 62.7 (CH_2CH_2), 49.5 (CH_2CH_2), 46.9 ($\underline{CH_2}CH_3$), 44.3 ($\underline{CH_2}CH_2$), 8.4 ($CH_2\underline{CH_3}$). Anal. calcd. for $C_{22}H_{27}N_5O \times 2HCl \times 3H_2O$ (M_r = 504.45): C 52.38, H 6.99, N 13.88; found: C 52.21, H 6.58, N 14.12.

2-{4-[2-(Diethylamino)ethoxy]-3-fluorophenyl}-5-(imidazolin-2-yl)-1*H*-benzo[*d*]imidazole dihydrochloride (l8b)

According to the above-mentioned procedure, from **13b** (150 mg; 0,63 mmol) compound **18b** was obtained as brown solid (157,0 mg; 63 %, m.p. = 205–207 °C). ^{1}H NMR (300 MHz, DMSO-d_6) (δ/ppm): 11.00 (1H, bs, NH), 10.90 (2H, s, CNH), 8.48 (1H, s, H4), 8.40–8.26 (2H, m, Ph), 8.00 (1H, dd, J = 8.6, 1.5 Hz, H6), 7.87 (1H, d, J = 8.6 Hz, H7), 7.52 (1H, t, J = 8.7 Hz, Ph), 4.64 (2H, t, J = 4.8 Hz, CH_2CH_2), 4.02 (4H, s, $\underline{CH_2}CH_2$), 3.67–3.52 (2H, m, CH_2CH_2), 3.32–3.14 (4H, m, $\underline{CH_2}CH_3$), 1.28 (6H, t, J = 7.2 Hz, $CH_2\underline{CH_3}$). ^{13}C NMR (75 MHz, DMSO-d_6) (δ/ppm): 165.1 (CNH), 153.1; 149.8 (d, J_{CF} = 245.0 Hz, Ph-q), 152.7 (C2), 148.3; 148.1 (d, J_{CF} = 10.7 Hz, Ph-q), 124.8; 124.8 (J_{CF} = 1.2 Hz, Ph), 123.4 (C6), 121.1; 121.0 (d, J_{CF} = 7.5 Hz, Ph-q), 116.5 (C4), 116.4 (C5), 115.5 (Ph), 115.3; 115.1 (d, J_{CF} = 20.7 Hz, Ph), 115.0 (C7), 63.9 (CH_2CH_2), 49.5 (CH_2CH_2), 47.3 ($\underline{CH_2}CH_3$), 44.3 ($\underline{CH_2}CH_2$), 8.5 ($CH_2\underline{CH_3}$). Anal. calcd. for $C_{22}H_{26}FN_5O \times 2HCl \times 1.5H_2O$ (M_r = 495.42): C 53.34, H 6.31, N 14.14; found: C 53.70, H 6.11, N 14.27.

2-{4-[2-(Diethylamino)ethoxy]-3-methoxyphenyl}-5-(imidazolin-2-yl)-1*H*-benzo[*d*]imidazole dihydrochloride (l8c)

According to the above-mentioned procedure, from **13c** (150 mg, 0.60 mmol) compound **18b** was obtained as yellow solid (183.0 mg, 75%, m.p. = 227–229 °C). ^{1}H NMR (300 MHz, DMSO-d_6) (δ/ppm): 10.92 (1H, bs, NH), 10.78 (2H, s, CNH), 8.43 (1H, d, J = 1.0 Hz, 4H), 8.12 (1H, d, J = 1.7 Hz, Ph), 8.01 (1H, dd, J = 8.4, 1.8 Hz, Ph), 7.95 (1H, dd, J = 8.6, 1.5 Hz, H6), 7.83 (1H, d, J = 8.5 Hz, H7), 7.27 (1H, d, J = 8.5 Hz, Ph), 4.62–4.36 (2H, m, CH_2CH_2), 4.03 (4H, s, $\underline{CH_2}CH_2$), 3.96 (3H, s, OCH_3), 3.59–3.38 (2H, m, CH_2CH_2), 3.32–3.20 (4H, m, $\underline{CH_2}CH_3$), 1.30 (6H, t, J = 7.2 Hz, $CH_2\underline{CH_3}$). ^{13}C NMR (75 MHz, DMSO-d_6) (δ/ppm): 165.1 (CNH), 153.6 (C2), 149.3 (Ph-q), 123.0 (Ph), 120.8 (C6), 117.3 (C5), 115.9 (C4), 114.5 (C7), 113.9 (Ph), 111.5 (Ph), 63.5 (CH_2CH_2), 56.2 (OCH_3), 49.5 (CH_2CH_2), 47.1 ($\underline{CH_2}CH_3$), 44.1 ($\underline{CH_2}CH_2$), 8.3 ($CH_2\underline{CH_3}$). Anal. calcd. for $C_{23}H_{29}N_5O_2 \times 2HCl \times 2H_2O$ (M_r = 516.46): C 53.49, H 6.83, N 13.56; found: C 53.11, H 6.33, N 13.89.

4.3. Anti-trypanosomal screening and cytotoxicity assays

4.3.1. Anti-trypanosomal screening

Bloodstream form *T. brucei* (strain 221) were grown in modified Iscove's medium, as described [77] and growth inhibition assays were performed using 96-well microtiter plates. The compound concentrations that inhibited growth by 50% (IC_{50}) and 90% (IC_{90}) were determined. Parasites were initially sub-cultured at 2.5×10^4 mL^{-1}, compounds were added at

range of concentrations, and the plates incubated at 37 °C. Resazurin was added after 48 h, the plates incubated for a further 16 h, and then read in a Spectramax plate reader. The data were analysed using GraphPad Prism. Each drug concentration was tested in triplicate.

4.3.2. L6 cell proliferation

For cytotoxicity assays, L6 cells (a rat myoblast line) were seeded into 96-well microtiter plates at 1 x 10^4 mL^{-1} in 200 μL of growth medium, and different compound concentrations were added. The plates were then incubated for 6 days at 37 ºC and 20 μL resazurin added to each well. After further 8 h incubation, the fluorescence was determined using a Spectramax plate reader, as outlined above.

4.4. DNA and RNA binding study

The UV/vis spectra were recorded on a Varian Cary 100 Bio spectrophotometer, CD spectra on JASCO J815 spectrophotometer and fluorescence spectra on a Varian Cary Eclipse spectrophotometer at 25 ºC using appropriate 1cm path quartz cuvettes.

Polynucleotides were purchased as noted: poly A–poly U, poly $(dAdT)_2$, poly $(dGdC)_2$ and calf thymus ctDNA (Sigma-Aldrich). Polynucleotides were dissolved in Na-cacodylate buffer, I = 0.05 mol dm^{-3}, pH = 7. The calf thymus ctDNA was additionally sonicated and filtered through a 0.45 mm filter [78]. Polynucleotide concentration was determined spectroscopically [79,80] as the concentration of phosphates. Spectrophotometric titrations were performed at pH = 7 (I = 0.05 mol dm^{-3}, sodium cacodylate buffer) by adding portions of polynucleotide solution into the solution of the studied compound for fluorimetric experiments and for CD experiments were done by adding portions of compound stock solution into the solution of polynucleotide. In fluorimetric experiments excitation wavelength of λ_{exc} = 317 and 320 nm was used to avoid the inner filter effect caused due to increasing absorbance of the polynucleotide. Emission was collected in the range λ_{em} = 330–700 nm. Values for K_s obtained by processing titration data by means of Scatchard equation [43], all have satisfactory correlation coefficients (>0.99). Thermal melting curves for DNA, RNA and their complexes with studied compounds were determined as previously described by following the absorption change at 260 nm as a function of temperature. Absorbance of the ligands was subtracted from every curve and the absorbance scale was normalized. T_m values are the midpoints of the transition curves determined from the maximum of the first derivative and checked graphically by the tangent method. The ΔT_m values were calculated subtracting T_m of the free nucleic acid from T_m of the complex. Every ΔT_m value here reported was the average of at least two measurements. The error in ΔT_m is ±0.5ºC.

4.5. Computational methods

Known inhibitors are retrieved from Chembl. Available X-ray structures of DNA complexes with small ligands were downloaded from the Protein data bank [51]. The ligand docking studies were carried out using Glide docking protocol with extra precision (XP) [81–84] within Schrödinger suite of software [85]. Binding poses were refined and binding energy was estimated using MM-GBSA [86–88] protocol and OPLS3 force-field with flexible residues distance being 5Å. Molecular dynamic simulations were carried out using Desmond software within the Schroedinger package [89–91]. Simulations were carried out at the room temperature for 20 ns. *In silico* ADME properties as well as structural parameters were calculated by ACD Percepta software [92].

4.6. In vitro ADME profiling

4.6.1. MDCKII-MDR1 permeability assay

MDCKII-hMDR1 cells were obtained from Solvo Biotechnology, Hungary. DMEM, Fetal bovine serum, Glutamax-100, Antibiotic/Antimycotic, DMSO, Dulbecco's phosphate buffer saline, MEM Non-essential amino acids were purchased from Sigma (St. Louis, MO, USA). Bi-directional permeability and P-glycoprotein substrate assessment were investigated in Madin-Darby canine epithelial cells with over-expressed human MDR1 gene (MDCKII-MDR1), coding for P-glycoprotein. Experimental procedures, as well as cell culture conditions, were the same as previously described [93]. Briefly, compounds (10 μM, 1% DMSO v/v) in duplicate were incubated at 37°C for 60 min with cell monolayer on 24-well Millicell inserts (Millipore, Burlington, MA, USA) without and with the P-glycoprotein inhibitor Elacridar (2 μM, International Laboratory, USA). Inhibition of P-glycoprotein was verified by amprenavir (Moravek Biochemicals Inc, Brea, CA, USA) and monolayer integrity by Lucifer yellow (Sigma, St. Louis, MO, USA). Compound concentrations were measured by LC-MS/MS and Lucifer yellow was measured on an Infinite F500 (Tecan, Männedorf, CH) using excitation of 485 nm and emission of 530 nm.

4.6.2. Metabolic stability

Mouse liver microsomes were obtained from Corning Life Sciences (Corning, USA). DMSO, nicotinamide adenine dinucleotide phosphate (NADP), glucose-6-phosphate, glucose-6-phosphate dehydrogenase, magnesium chloride, propranolol, caffeine, diclofenac, phosphate buffer saline (PBS) were purchased from Sigma (St. Louis, MO, USA). Acetonitrile (ACN) and methanol (MeOH) were obtained from Merck (Darmstadt, Germany). Testosterone was

purchased from Steraloids (Newport, RI, USA). Metabolic stability was assessed in mouse liver microsomes. Compounds (final concentration of 1 µM, 0.03% DMSO v/v) were incubated in duplicate in phosphate buffer (50 mM, pH 7.4) at 37°C together with mouse liver microsomes in the absence and presence of the NADPH cofactor (0.5 mM nicotinamide adenine dinucleotide phosphate, 5 mM glucose-6-phosphate, 1.5 U/mL glucose-6-phosphate dehydrogenase and 0.5 mM magnesium chloride). Incubation and sampling was performed on a Freedom EVO 200 (Tecan, Männedorf, CH) at 0.3, 10, 20, 30, 45 and 60 min. The reaction was quenched using 3 volumes of a mixture of ACN/MeOH (2:1) containing internal standard (diclofenac), centrifuged and supernatants were analyzed using LC-MS/MS.

Metabolic activity of microsomes was verified by simultaneous analysis of several controls including testosterone, propranolol and caffeine. The in vitro half-life ($t_{1/2}$) was calculated using GraphPad Prism non-linear regression of % parent compound remaining versus time. In vitro clearance, expressed as µL/min/mg, was estimated from the in vitro half-life ($t_{1/2}$), and normalized for the protein amount in the incubation mixture and assuming 52.5 mg of protein per gram of liver.

4.6.3. LC-MS/MS analysis

All samples were quantified using tandem mass spectrometry coupled to liquid chromatography. Samples were analysed on a Sciex API4000 Triple Quadrupole Mass Spectrometer (Sciex, Division of MDS Inc., Toronto, Canada) coupled to a Shimadzu Nexera X2 UHPLC frontend (Kyto, Japan). Samples were injected onto a UHPLC column (HALO2 C18, 2.1x20 mm, 2 µm or Luna Omega 1.6 µm Polar C18 100A, 30x2.1 mm) and eluted with a gradient at 50 °C. The mobile phase was composed of acetonitrile/water mixture (9/1, with 0.1 % formic acid) and 0.1 % formic acid in deionized water. The flow rate was 0.7 mL/min and under gradient conditions, leading to a total run time of 1.5–2 min. Positive ion mode with turbo spray, an ion source temperature of 550 °C and a dwell time of 150 ms were utilized for mass spectrometric detection. Quantitation was performed using multiple reaction monitoring (MRM) at the specific transitions for each compound.

Declaration of interest

Declaration of interest: none.

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