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Original article

Synthesis and cytotoxicity properties of amiodarone analogues

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Abstract

Amiodarone (AMI) is a potent antiarrhythmic agent; however, its clinical use is limited due to numerous side effects. In order to investigate the structure–cytotoxicity relationship, AMI analogues were synthesized, and then, using rabbit alveolar macrophages, were tested for viability and for the ability to interfere with the degradation of surfactant protein A (SP-A) and with the accumulation of an acidotropic dye. Our data revealed that modification of the diethylamino-β-ethoxy group of the AMI molecule may affect viability, the ability to degrade SP-A and vacuolation differently. In particular, PIPAM (**2d**), an analogue with a piperidyl moiety, acts toward the cells in a similar manner to AMI, but is less toxic. Thus, it would be possible to reduce the cytotoxicity of AMI by modifying its chemical structure.

Keywords: Amiodarone; Amiodarone analogues; Cytotoxicity; Endocytic pathway

1. Introduction

Fifty years ago, during the search for potent coronary dilators, the scientists of Labaz laboratories modified the chemical structure of khellin (Fig. 1), which is a natural product with known coronary dilator property. Further research led to the discovery of amiodarone (AMI) as an antianginal drug [1]; its antidysrhythmic activity was only observed later [2]. At present, AMI is one of the most potent antiarrhythmic agents in the treatment of ventricular and supraventricular arrhythmias [3,4]. However, AMI therapy is accompanied by many undesired effects [5–8]. The mechanism of toxicity is multifactorial and may result from the accumulation of AMI itself (and/or metabolites, including iodine), altered cellular function, free radicals overproduction, etc. [9,10]. Furthermore, the long elimination half-life (months after oral administration) of the drug complicates clinical decisions, when a change in treatment strategy is desired. Efforts have been made, therefore, to modify the chemical structure of AMI in order to obtain a better therapeutic profile [11–14].

Among side effects of AMI, pulmonary toxicity is probably the most significant and potentially life-threatening effect associated with AMI use. In humans, this incidence was approximately 6%, with a mortality rate estimated at 5–10% of affected patients [15]. Pathologically, AMI-induced pulmonary toxicity (AIPT) is characterized by the accumulation of phospholipids (phospholipidosis) in the lungs, alveolitis, with fibrosis developing in some patients. The principal response of lung phospholipidosis is the ‘‘foamy’’ lung, which is due to the appearance of large intra-alveolar cells. These

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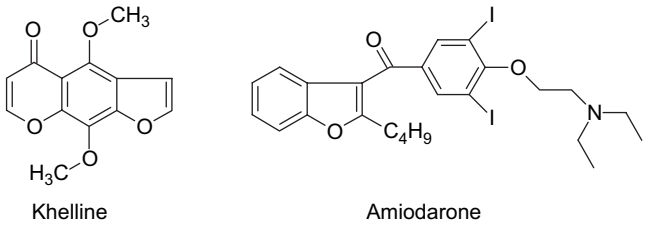


Fig. 1. Chemical structure of khellin and amiodarone.

cells, which appear to originate from alveolar macrophases, contain lysosomally derived lamellar inclusions [16,17]. Alveolar macrophases were previously used in AMI lung toxicity investigations [18,19].

This work aims to look for the way how to minimize the cytotoxicity of AMI. For this goal, a group of new AMI derivatives with modified ethoxydiethylamine side chain was synthesized, and their effects on alveolar macrophages [viability, release of lactate dehydrogenase (LDH), degradation of surfactant protein A (SP-A) and uptake of an acidotropic dye] were investigated.

2. Results and discussion

2.1. Chemistry

The starting product B2 (**1**) can be obtained with high purity by recrystallization from ethyl carbonate. From this compound, other target compounds may be prepared by condensing it with the corresponding halogenated alkylamine-products. For this step, using an excess of halogenated reagents and always keeping the temperature of the reaction under 90 °C are recommended.

The analogues MeAM (**2a**), DIPAM (**2b**), PYRAM (**2c**), PIPAM (**2d**), and MOPAM (**2e**) have chemical structures that are very close to that of AMI (diiodo-moiety linking to an ethoxy-*tert*-amino side chain). The alkoxyamine chain of PPAM (**2f**) is one $-CH_2$ unit longer than that of MeAM (**2a**).

2.2. Physicochemical properties of AMI analogues: aqueous solubility, liposolubility and interaction with plasma membranes using chromatography

The published data support the notion that AMI is sequestered in plasma membranes and, hence, influence its fluidity and also enzyme activity [20]. This prompted us to use the immobilized artificial membrane (IAM) to study the affinity ($\log D_{IAM7.4}$) of AMI derivatives toward plasma membranes [21], and octadecyl silane (ODS) reversed-phase HPLC to quantify their lipophilicity ($\log D_{ODS7.4}$) [22,23]. Additionally, the aqueous solubility of AMI derivatives having diverse polar moieties was also measured at 22 °C in 5 mM phosphate buffer pH 7.4, and pH 5.0 (around the pH of lysosomes) using the published method [24]. Our data (Table 1) suggest that: (i) the modification of the alkoxyamine moiety of AMI, as described herein, does not induce a great change in the physicochemical properties of the origin molecule; (ii) the affinity of AMI analogues toward plasma membranes is dominated by a partitioning mechanism, however, the charge interaction between solutes and phospholipids is not negligible (unpublished results).

Table 1
Physicochemical parameters of amiodarone analogues

Compound	Solubility[a] at pH 5.0 (μM)	Solubility[a] at pH 7.4 (μM)	$\log D_{ODS7.4}$[b]	$\log D_{IAM7.4}$[c]
AMI	2.04	0.016	7.91	4.23
2a MeAM	2.21	0.051	7.15	3.64
2b DIPAM	0.23	0.014	8.74	4.58
2c PYRAM	1.86	0.049	7.82	4.23
2d PIPAM	2.22	0.023	8.04	4.28
2e MOPAM	0.17	0.015	6.15	3.36
2f PPAM	1.04	0.212	7.98	3.56

The result is the mean of triplicates.
[a] Solubility of amiodarone analogues at 22 °C in 5 mM K_2HPO_4 adjusted to pH 5.0 and pH 7.4 with dilute HCl. Methanol was used as cosolvent. See Ref. [24].
[b] Lipophilicity as measured by octadecyl silane (ODS)-RP-HPLC in 0.01 M phosphate buffer at pH 7.4 and methanol. See Ref. [23].
[c] Membrane affinity as measured by IAM-HPLC at 37 °C in 0.01 M K_2HPO_4 adjusted to pH 7.4 with dilute HCl. Acetonitrile was used as cosolvent. See Ref. [21].

2.3. Effect of AMI analogues on alveolar macrophages

AMI main metabolite monodesethylamiodarone and other analogues, including MeAM (**2a**), have been used for investigating their effect on the degradation of SP-A by alveolar macrophages in our previous studies [19]. We completed this study to investigate the effect of five newly synthesized derivatives [DIPAM (**2b**), PYRAM (**2c**), PIPAM (**2d**), MOPAM (**2e**), and PPAM (**2f**)]. Using trypan blue exclusion as an index of viability, it was observed that AMI, PYRAM (**2c**), and PPAM (**2f**) decreased macrophage viability at high concentrations (50 μM), while the other analogues had no effect (Fig. 2). Considering the release of LDH as a measure of cell damage, it appears that PPAM (**2f**) and PYRAM (**2c**), having similar toxicity

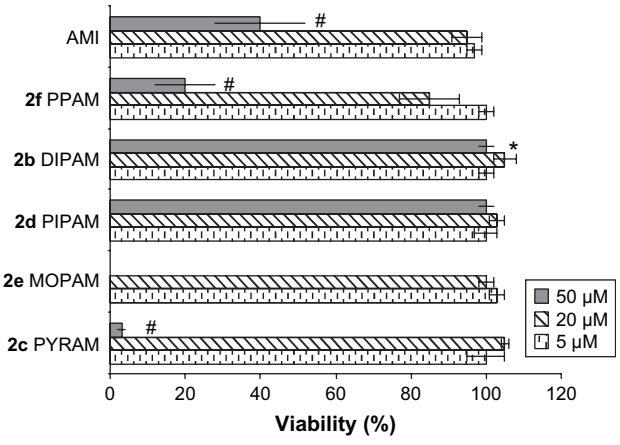


Fig. 2. Effect of amiodarone analogues on viability of alveolar macrophages analyzed by trypan blue exclusion. Mean ± SE; $n = 7–13$. *Different from amiodarone at same concentration, #different from other concentrations of the same drug (by ANOVA).

behaviors, were the most toxic among the compounds tested (Fig. 3). Using the degradation of SP-A as an index of integrity of the endocytic pathway, it appears that AMI, PYRAM (**2c**), PIPAM (**2d**), and PPAM (**2f**) all inhibited the degradation of SP-A to a similar extent, while DIPAM (**2b**) and MOPAM (**2e**) had no effect (Fig. 4).

Furthermore, control macrophages accumulated Lysosensor in small vesicles clustered near the nucleus, but also displayed a diffuse staining of the cytoplasm (Fig. 5). A similar pattern of fluorescence was seen in cells treated with B2 (**1**) and MOPAM (**2e**). It was previously shown that B2 (**1**) has no effect on the degradation of SP-A [19]. In contrast, macrophages treated with AMI, PYRAM (**2c**), PIPAM (**2d**), and PPAM (**2f**) accumulated Lysosensor in a few very large vacuoles, frequently located at the periphery of the cell, with less staining of the cytoplasm (Fig. 5). Macrophages treated with DIPAM (**2b**) accumulated Lysosensor into small vesicles located near the nucleus, as in control cells, displayed an intense staining of the cytoplasm, and presented huge blisters at their periphery (Fig. 5).

Considering the effects of analogues on viability, release of LDH, degradation of SP-A and uptake of Lysosensor, it appears that analogues causing the accumulation of Lysosensor into large vacuoles [AMI, PYRAM (**2c**), PIPAM (**2d**), and PPAM (**2f**)] were the ones that inhibit most the degradation of SP-A, indicating that disordering of vesicular traffic and inhibition of proteolysis may be interconnected (compare Figs. 4 and 5). These findings reinforce the view that high solubility at neutral pH may favor interaction with the plasma membrane, while high solubility at pH 5.0 may elevate the accumulation in the lumen of acidic organelles. It is tempting to speculate that accumulation into endosomes could induce swelling by osmosis. It should be noticed, however, that swelling might also be due to the increased biogenesis, decreased budding or increased fusion of the limiting membranes with other organelles, processes that are under the control of diverse gene products and lipids [25]. Thus, the mechanism by which AMI and some AMI analogues cause swelling of late endosomes remains unclear.

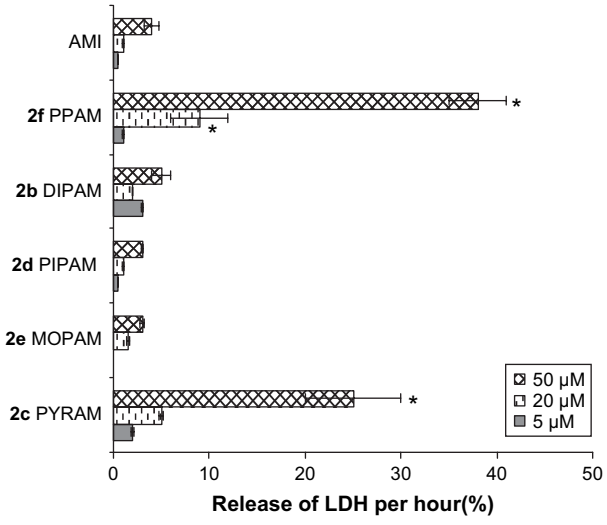


Fig. 3. Effect of amiodarone analogues on the release of lactate dehydrogenase (LDH) by alveolar macrophages. Values are expressed as a percentage of total LDH released per hour. Mean ± SE; $n = 7-10$. *Different from amiodarone at same concentration (by ANOVA).

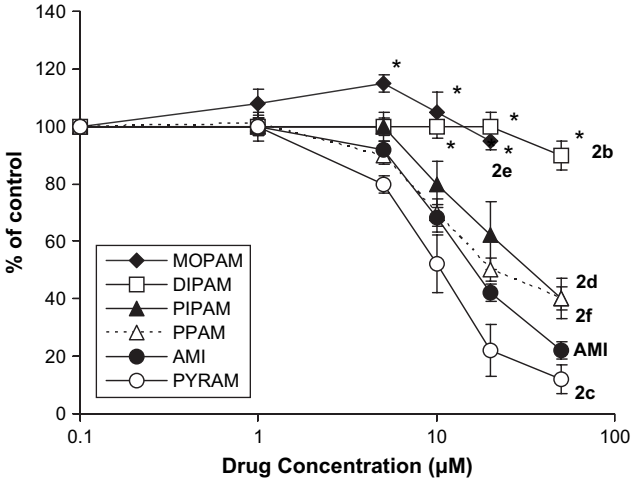


Fig. 4. Effects of amiodarone analogues on the degradation of SP-A by alveolar macrophages. Suspensions of 10^6 cells in 1 ml ringer buffered albumin (RBA) were incubated with different drugs for 1 h, then 1 µg of ^{125}I-SP-A was added, and the incubation was continued for further 2 h. At the end, TCA-solute radioactivity was counted. Data are presented as a percentage of degradation of control cells. Mean ± SE; $n = 3-11$. *Different from amiodarone at same concentration (by ANOVA).

Among the analogues studied, DIPAM (**2b**), PYRAM (**2c**), and PPAM (**2f**) are therefore more toxic than AMI, whereas PIPAM (**2d**), a piperidyl derivative of AMI, acts on alveolar macrophages in the same manner as AMI, although appears to be less toxic. It is also clear that lipophilicity, or partition into plasma membrane do not, alone or in combination, help in predicting the toxicity of AMI analogues toward alveolar macrophages.

3. Conclusion

In order to look for new derivatives of AMI, having less toxicity, a series of AMI analogues were synthesized and tested *in vitro*. Our data suggest that modifying the bulk of the ethoxy-*tert*-amino moiety of AMI molecule, as described in the current studies, does not change much the physicochemical properties, such as lipophilicity, or affinity for membranes. However, some of these modifications strongly influence the effects on cells, and it appears that the AMI molecule may be modified so as to maintain the effects on the endocytic pathway, in particular the ability to inhibit proteolysis, but with less *in vitro* toxicity.

4. Experimental

All melting points are uncorrected. ^{1}H, ^{13}C, and inverse gated ^{13}C NMR spectra were recorded on a Bruker ARX-300 spectrometer (operating at 300.13 MHz for ^{1}H and at

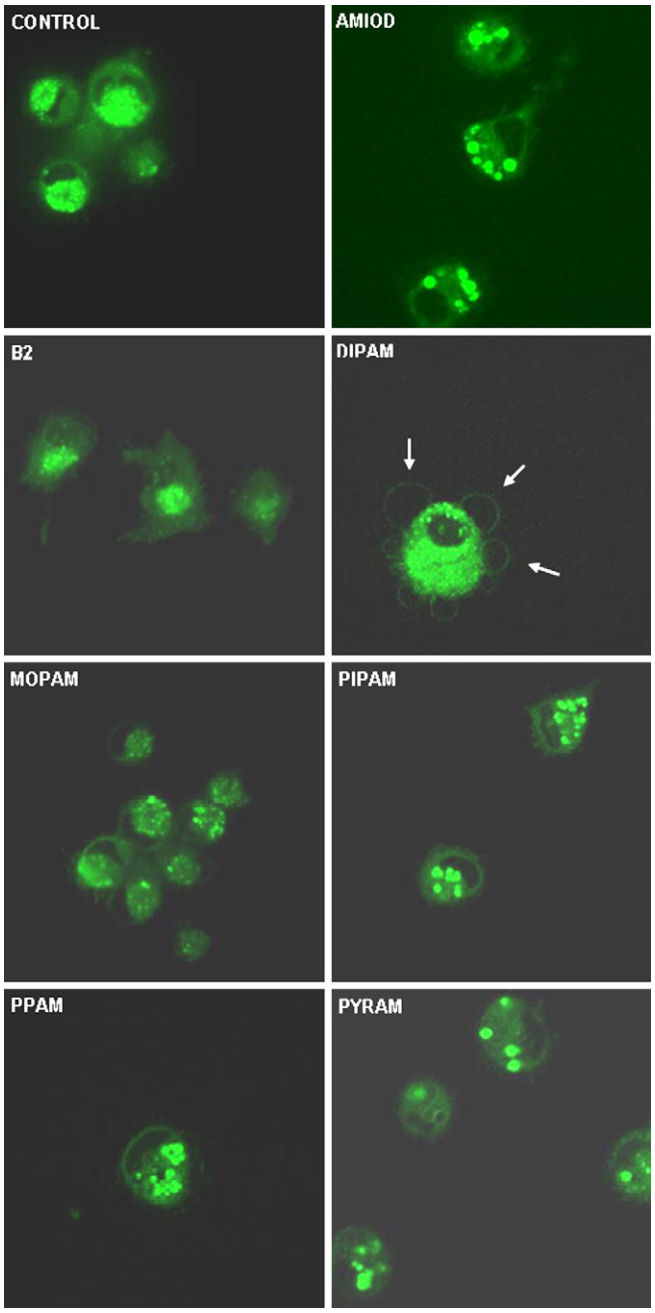


Fig. 5. Effect of amiodarone analogues on the distribution of Lysosensor Green DND-189 after uptake by alveolar macrophages. Cells were incubated for 16 h with 10 μM of each test compound, exposed for 20 min to 1 μg/ml Lysosensor and then examined by means of confocal microscopy. Arrows: blisters formed during incubation with DIPAM (**2b**).

75.47 MHz for ^{13}C) at 298 K. The samples were, unless otherwise stated, measured in d_6-DMSO, and tetramethylsilane was used as an internal reference. The electron impact (EI)-mass spectra (70 eV) were recorded on a Finnigan MAT95 instrument and the electrospray ionization (ESI)-mass spectra on a Bruker EsquireLC instrument. The samples, recorded using the latter method, were dissolved in MeOH at 10^{-4} M, and the spectra recorded at a 4 μl per min continuous flow in positive or negative mode. All solvents were of analytical grade quality and were used without further purification unless otherwise stated and dried using standard methods if needed. DMF was stored over 4 Å molecular sieve under nitrogen. Flash chromatography: silica gel Merck 60 (40–60 μm, 230–400 mesh). Thin layer chromatography (TLC): Merck precoated silica gel 60-F_{254} plates; detection by UV at 254 nm.

4.1. Chemistry

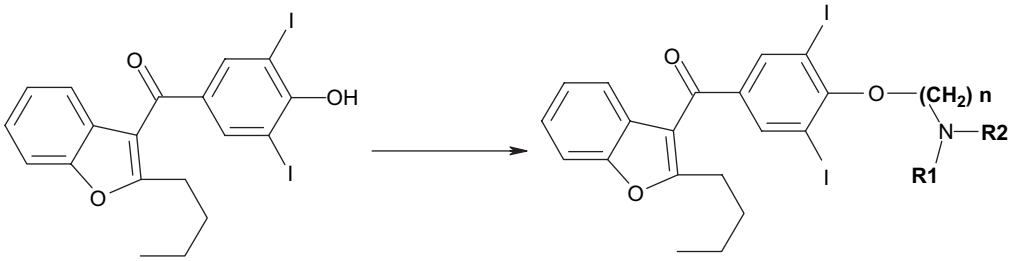
The AMI analogues were prepared from (2-butyl-benzofuran-3-yl)-(4-hydroxy-3,5-diiodophenyl)-methanone (B2, **1**), which had been synthesized previously [26], by condensing with the corresponding halogenated compounds (Scheme 1). The synthesis and analytical data of MOPAM (**2e**) were reported in our previous communication [27].

*4.1.1. (2-Butyl-benzofuran-3-yl)-(4-hydroxy-3, 5-diiodophenyl)-methanone (**1**, B2)*

This compound was prepared previously (yield 60%) [26]. The data supporting its chemical structure are reported herein; mp 146.4–146.9 °C; NMR: δ_H [300 MHz] 0.85 (t, *J* 7.3 Hz, 3H, CH_3), 1.27 (hex, *J* 7.4 Hz, 2H, CH_2), 1.70 (quin, *J* 7.3 Hz, 2H, CH_2), 2.77 (t, *J* 7.7 Hz, 2H, CH_2), 7.25–7.37 (m, 2H, aromatics), 7.44 (d, *J* 7.7 Hz, H-4), 7.64 (d, *J* 8.0 Hz, H-7), 8.10 (s, 2H, H-2′ and H6′), 10.44 (br s, 1H, OH); δ_C [75.47 MHz] 13.4 (CH_3), 21.9 (CH_2), 27.4 (CH_2), 29.3 (CH_2), 86.1 (C-3′ and C-5′), 111.1, 115.6, 120.7, 123.8, 124.7, 126.3, 134.0, 140.0 (H-C2′ and H-C6′), 153.0, 159.6, 164.4, 186.9 (CO); EI-MS (70 eV) *m/z* (%) 546 ($M^{\bullet+}$, 100), 517 (33), 504 (11), 503 (7), 420 (4), 390 (6), 376 (8), 375 (7), 373 (13), 263 (20), 250 (7), 249 (8), 221 (8), 201 (8).

*4.1.2. (2-Butyl-benzofuran-3-yl)-4-[2-(dimethylamino-ethoxy)-3,5-diiodophenyl]-methanone (**2a**, MeAM)*

The cytotoxicity of MeAM (**2a**) was previously investigated [19], its synthesis is reported herein as standard procedure. To a mixture of B2 (**1**) (2 g, 3.66 mmol) and K_2CO_3 (3.45 g, 25 mmol) in toluene/water (2:1 v/v, total volume: 75 ml) heated to 55–60 °C was added in small portions (about 0.2 g) *N*-dimethyl-2-chloroethylamine hydrochloride (2.6 g, 20 mmol; Aldrich, 9471 Buchs, Switzerland). After the addition, the temperature was raised to reach reflux over 30 min. The yellow color of B2 (**1**) disappeared. The reaction was refluxed for 1 h additionally, and the phases were separated quickly using a separation funnel at 60 °C. The toluene phase was then washed three times with 25 ml water at this temperature. The organic phase was evaporated to dryness. The residue was suspended in 10 ml 5% NH_3, and MeAM (**2a**) was extracted three times with 15 ml toluene. The organic phase was separated by means of centrifugation and evaporated to dryness under reduced pressure. Then, 2 ml 10 N HCl and 15 ml of toluene were added, and the liquids were taken off under reduced pressure at 80 °C. A white solid was obtained after three additional treatments with 10 ml toluene. The residue was then crystallized from toluene and gave 1.43 g (60%) of analytically pure MeAM·HCl: mp 189.5–189.7 °C; crystallized form of MeAM (**2a**) base may also be obtained: mp



Compound (yield in %)		n	R1	R2	Reagent
2a	MeAM (72)	2	CH_3	CH_3	Cl-CH_2-CH_2-N(CH_3)$_2$
2b	DIPAM (62)	2	$CH(CH_3)_2$	$CH(CH_3)_2$	Cl-CH_2-CH_2-N[$CH(CH_3)_2$]$_2$
2c	PYRAM (70)	2			Cl-CH_2-CH_2-
2d	PIPAM (64)	2			Cl-CH_2-CH_2- 
2e	MOPAM (65)	2			Cl-CH_2-CH_2- 
2f	PPAM (60)	3	CH_3	CH_3	Cl-CH_2-CH_3-CH_2-N=(CH_3)$_2$

Scheme 1. Synthesis of amiodarone (AMI) analogues.

89–90 °C. NMR: δ_H [300 MHz] 0.84 (t, *J* 7.3 Hz, 3H, CH_3), 1.24 (hex, *J* 7.4 Hz, 2H, CH_2), 1.69 (quin, *J* 7.4 Hz, 2H, CH_2), 2.49–2.51 (m, 3H, CH_3), 2.73 (t, *J* 7.7 Hz, 2H, CH_2), 2.82, (t, *J* 5.9 Hz, 2H, CH_2), 3.29–3.31 (m, 3H, CH_3), 4.08 (t, *J* 5.9 Hz, 2H, CH_2), 7.27–7.39 (m, 2H, aromatics), 7.48 (d, *J* 7.7 Hz, H-4), 7.65 (d, *J* 8.1 Hz, H-7), 8.16 (s, 2H, H-2′ and H6′); δ_C [75.47 MHz] 13.3 (CH_3), 21.8 (CH_2), 27.4 (CH_2), 29.4 (CH_2), 45.4 (N-(CH_3)$_2$), 58.2 (CH_2), 71.1 (CH_2), 92.9 (C-3′ and C-5′), 111.1, 115.5, 120.7, 123.8, 124.8, 126.0, 137.9, 139.7 (H-C2′ and H-C6′), 153.0, 161.0, 165.3, 187.3 (CO); ESI-MS $[M+H]^+$ *m/z* (%) = 618 (100).

Using the corresponding chloroalkoxylamine hydrochloride compounds purchased from Aldrich, 9471 Buchs, Switzerland, DIPAM (**2b**), PYRAM (**2c**), PIPAM (**2d**), MOPAM (**2e**), and PPAM (**2f**) were prepared in a similar manner (Scheme 1).

*4.1.3. (2-Butyl-benzofuran-3-yl)-4-[2-(diisopropylamino) ethoxy]-3,5-diiodophenyl-methanone·hydrochloride (**2b**, DIPAM)*

Mp 146.8–148.7 °C; NMR: δ_H [300 MHz] 0.85 (t, *J* 7.3 Hz, 3H, CH_3), 1.27 (hex, *J* 7.4 Hz, 2H, CH_2), 1.40 (d, *J* 6.3 Hz, 6H, CH_3), 1.42 (d, *J* 6.3 Hz, 6H, CH_3), 1.70 (quin, *J* 7.4 Hz, 2H, CH_2), 2.74 (t, *J* 7.7 Hz, 2H, CH_2), 3.58–3.66 (m, 2H, CH_2), 3.80–3.89 (m, 2H, CH_2), 4.40 (t, *J* 6.9 Hz, 2H, CH_2), 7.28–7.39 (m, 2H, aromatics), 7.48 (d, *J* 7.8 Hz, H-4), 7.66 (d, *J* 7.8 Hz, H-7), 8.19 (s, 2H, H-2′ and H6′), 10.2 (br s, 1H, HN^+); δ_C [75.47 MHz] 13.4 (CH_3), 16.7 (CH_3), 18.0 (CH_3), 21.9 (CH_2), 27.5 (CH_2), 29.3 (CH_2), 30.6 (CH), 45.3 (CH_2), 54.5 (CH), 68.1 (CH_2), 92.1 (C-3′ and C-5′), 111.2, 115.6, 120.8, 123.9, 124.9, 126.1, 138.7, 139.8 (H-C2′ and H-C6′), 153.1, 160.0, 165.6, 187.3 (CO); ESI-MS $[M+H]^+$ *m/z* (%) = 674 (100).

*4.1.4. [4-(2-Butyl-benzofuran-3-yl)-[3,5-diiodophenyl-4-(2-pyrrolidin-1-yl-ethoxy)phenyl]-methanone· hydrochloride (**2c**, PYRAM)*

Mp 93.6–95.4 °C; NMR: δ_H [300 MHz] 0.84 (t, *J* 7.3 Hz, 3H, CH_3), 1.26 (hex, *J* 7.4 Hz, 2H, CH_2), 1.69 (quin, *J* 7.4 Hz, 2H, CH_2), 1.92–2.08 (m, 4H, CH_2), 2.73 (t, *J* 8.0 Hz, 2H, CH_2), 3.22–3.32 (m, 2H, CH_2), 3.72–3.74 (m, 4H, CH_2), 4.34–4.37 (m, 2H, CH_2), 7.26–7.39 (m, 2H, aromatics), 7.46 (d, *J* 7.7 Hz, H-4), 7.64 (d, *J* 8.1 Hz, H-7), 8.18 (s, 2H, H-2′ and H6); δ_C [75.47 MHz] 13.3 (CH_3), 21.8 (CH_2), 22.6 (2CH_2), 27.4 (CH_2), 29.2 (CH_2), 53.1 (CH_2), 53.5 (2CH_2), 68 (CH_2), 92.1 (C-3′ and C-5′), 111.1, 115.5, 120.7, 123.8, 124.8, 126.0, 138.5, 139.7 (H-C2′ and H-C6′), 153.0, 160.0, 165.5, 167.3 (CO); EI-MS (70 eV) *m/z* (%) = 643 ($M^{\bullet+}$, <1%), 546 (4), 517 (5), 516 (17), 420 (2), 201 (3), 97 (5), 84 (6), 389 (100), 55 (3).

*4.1.5. [4-(2-Butyl-benzofuran-3-yl)-[3,5-diiodophenyl-4-(2-piperidinoethoxy)phenyl]-methanone·hydrochloride (**2d**, PIPAM)*

Mp 131.3–172.0 °C; NMR: δ_H [300 MHz] 0.845 (t, *J* 7.3 Hz, 3H, CH_3), 1.27 (hex, *J* 7.4 Hz, 2H, CH_2), 1.70 (quin,

J 7.4 Hz, 2H, CH_2), 1.76–1.91 (m, 6H, CH_2), 2.74 (t, *J* 7.8 Hz, 2H, CH_2), 3.08–3.20 (m, 2H, CH_2), 3.65–3.69 (m, 4H, CH_2), 4.40–4.30 (m, 2H, CH_2), 7.27–7.48 (m, 2H, aromatics), 7.47 (d, *J* 7.5 Hz, H-4), 7.66 (d, *J* 8.0 Hz, H-7), 8.18 (s, 2H, H-2′ and H6′); δ_C [75.47 MHz] 13.3 (CH_3), 21.1 (CH_2), 21.8 (CH_2), 22.3 [$(CH_2)_2$], 27.4 (CH_2), 29.3 (CH_2), 52.5 [$(CH_2)_2$], 55.4 (CH_2), 67.1 (CH_2), 92.1 (C-3′ and C-5′), 111.1, 115.5, 120.7, 123.8, 124.8, 126.0, 138.5, 139.7 (H-C2′ and H-C6′), 153.0, 160.2, 165.6, 187.3 (CO); ESI-MS (70 eV) *m/z* (%) = 658 (100); EI-MS (70 eV) *m/z* (%) = 657 ($M^{\bullet+}$, <1%), 546 (3), 530 (6), 420 (2), 201 (2), 147 (3), 146 (2), 112 (2), 98 (100), 96 (3), 91 (2), 55 (4).

4.1.6. (2-Butyl-benzofuran-3-yl)-4-[3-(dimethylamino-propoxy)-3,5-diiodophenyl]-methanone·hydrochloride (**2f**, PPAM)

Mp 158.1–163.8 °C; NMR: δ_H [300 MHz] 0.85 (t, *J* 7.4 Hz, 3H, CH_3), 1.27 (hex, *J* 7.4 Hz, 2H, CH_2), 1.69 (quin, *J* 7.4 Hz, 2H, CH_2), 2.30–2.36 (m, 2H, CH_2), 2.73 (t, *J* 7.7 Hz, 2H, CH_2), 2.82, (s, 6H, CH_3), 3.34–3.42 (m, 2H, CH_2), 4.09 (t, *J* 5.7 Hz, 2H, CH_2), 7.27–7.40 (m, 2H, aromatics), 7.48 (d, *J* 7.7 Hz, H-4), 7.65 (d, *J* 7.9 Hz, H-7), 8.17 (s, 2H, H-2′ and H6′); δ_C [75.47 MHz] 13.3 (CH_3), 21.8 (CH_2), 24.7 (CH_2), 27.4 (CH_2), 29.2 (CH_2), 41.9 [N-$(CH_3)_2$], 54.0 (CH_2), 70.1 (CH_2), 92.1 (C-3′ and C-5′), 111.1, 115.5, 120.7, 123.8, 124.8, 126.0, 138.2, 139.6 (H-C2′ and H-C6′), 153.0, 160.3, 165.4, 187.3 (CO); ESI-MS $[M+H]^+$ *m/z* (%) = 632 (100).

4.2. Measurement of physicochemical properties: aqueous solubility, lipophilicity and interaction with plasma membranes using chromatography

The affinity of AMI analogues toward plasma membrane ($\log D_{IAM7.4}$) was investigated using immobilized artificial membrane (IAM), and acetonitrile as organic modifier [21]. The lipophilicity ($\log D_{ODS7.4}$) of AMI analogues was measured by means of octadecyl silane (ODS) reversed-phase HPLC, and methanol as organic modifier [22,23]. The solubility of tested compounds at 22 °C in 5 mM phosphate buffer pH 7.4, and pH 5.0 (around the pH of lysosomes) was measured by the described method [24]. The details of these experiments are reported elsewhere (to be published). The physicochemical parameters of tested compounds are expressed as the mean of three independent measurements (Table 1). The precision of estimates was <8%.

4.3. Cytotoxicity of AMI analogues

4.3.1. Trypan blue exclusion, release of LDH, and degradation of SP-A tests

SP-A was isolated from the surfactant obtained from a patient with alveolar proteinosis and labeled with $Na^{125}I$ to a specific activity of 400–600 cpm/ng, as reported previously [28]. Alveolar macrophages were obtained from rabbits. The animals were housed according to the rules set forth by the University of Padova for care and use of animals for scientific purposes. The isolated macrophages were suspended in ringer buffered albumin (RBA; containing 145 mM NaCl, 5 mM KCl, 1.0 mM $MgCl_2$, 2.5 mM Na_2HPO_4, 10 mM HEPES, 10 mM glucose, 1.0 mg/ml bovine albumin, pH 7.4) at a concentration of 10^6 cells/ml and used as suspensions after adhesion to Falcon plates (Becton Dickinson Labware, Meylan, France).

To study the effect of analogues on the ability of macrophages to degrade SP-A, alveolar macrophages (10^6 cells suspended in 1 ml of RBA) were incubated for 1 h at 37 °C in the presence of different analogues added in 1 μl of MDSO (final concentration 0–50 μM). Then 1 μg of ^{125}I-SP-A was added, and the incubation was continued for 1 h. Finally, the radioactivity present in medium plus cells was measured. Degradation of SP-A is presented as the percentage of the degradation measured in control cells after subtraction of the time 0 value (0.3–0.5% of added radioactivity).

The effect of analogues on trypan blue exclusion and on release of LDH by alveolar macrophages was measured as described previously [19].

4.3.2. Uptake of Lysosensor Green DND-189

Macrophages adhering to glass cover slides were incubated for 16 h at 37 °C in the presence of 2–10 μM test compounds before being loaded for 20 min at 37 °C with 1 μmol/l LysoSensor Green DND-189, which is an acidotropic dye that accumulates into acidic organelles as a result of protonation, and its fluorescence intensity is proportional to acidity [29]. Confocal images were obtained using a Nikon Eclipse TE300 inverted microscope equipped with the NIH Image J 1.32J (National Institutes of Health, Bethesda, USA).

4.4. Statistical analysis

Data are presented as mean ± SE. Differences between groups were analyzed by means of ANOVA, using the Dunnet's test as the post hoc test for data normally distributed and the Dunn's test for the normally distributed. The level of significance accepted was 5%.

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References

[1] R. Charlier, G. Deltour, R. Tondeur, F. Binon, Arch. Int. Pharmacodyn. Ther. 139 (1962) 255–264.
[2] J. Van Schepdael, H. Solvay, Presse Med. 78 (1970) 1849–1850.
[3] B.A. Weinberg, W.M. Miles, L.S. Klein, J.E. Bolander, R.E. Dusman, M.S. Stanton, J.J. Heger, C. Langefeld, D.P. Zipes, Am. Heart J. 125 (1993) 109–120.
[4] B.N. Singh, Am. J. Cardiol. 84 (1999) 3R–10R.
[5] R.N. Fogoros, K.P. Anderson, R.A. Winkle, C.D. Swerdlow, J.W. Mason, Circulation 68 (1983) 88–94.
[6] J.J. Heger, E.N. Prystowsky, D.P. Zipes, Am. Heart J. 106 (1983) 931–935.
[7] B. Rigas, L.E. Rosenfeld, K.W. Barwick, R. Enriquez, J. Helzberg, W.P. Batsford, M.E. Josephson, C.A. Riely, Ann. Intern. Med. 104 (1986) 348–351.

[8] G. Dickstein, S. Amikam, E. Riss, D. Barzilai, Am. J. Med. Sci. 288 (1984) 14–17.
[9] P.J. Podrid, Ann. Intern. Med. 122 (1995) 689–700.
[10] A. Vereckei, A. Blazovics, I. Gyorgy, E. Feher, M. Toth, G. Szenasi, A. Zsinka, G. Foldiak, J. Feher, J. Cardiovasc. Electrophysiol. 4 (1993) 161–177.
[11] W. Sun, J.S. Sarma, B.N. Singh, Circulation 100 (1999) 2276–2281.
[12] B. Carlsson, B.N. Singh, M. Temciuc, S. Nilsson, Y.L. Li, C. Mellin, J. Malm, J. Med. Chem. 45 (2002) 623–630.
[13] O.F. Sharifov, V.V. Fedorov, G.G. Beloshapko, A.V. Yushmanova, L.V. Rosenshtraukh, J. Cardiovasc. Pharmacol. 38 (2001) 706–714.
[14] A. Manning, V. Thisse, D. Hodeige, J. Richard, J.P. Heyndrickx, P. Chatelain, J. Cardiovasc. Pharmacol. 25 (1995) 252–261.
[15] W.J. Martin 2nd, E.C. Rosenow 3rd, Chest 93 (1988) 1242–1248.
[16] W.J. Martin 2nd, J.E. Standing, J. Pharmacol. Exp. Ther. 244 (1988) 774–779.
[17] F.R. Costa-Jussa, B. Corrin, J.M. Jacobs, J. Pathol. 144 (1984) 73–79.
[18] C.L. Ogle, M.J. Reasor, Toxicology 62 (1990) 227–238.
[19] D. Quaglino, H.R. Ha, E. Duner, D. Bruttomesso, L. Bigler, F. Follath, G. Realdi, A. Pettenazzo, A. Baritussio, Am. J. Physiol. Lung Cell. Mol. Physiol. 287 (2004) L438–L447.
[20] P. Chatelain, R. Laruel, M. Gillard, Biochem. Biophys. Res. Commun. 129 (1985) 148–154.
[21] S. Ong, X. Qui, C. Pidgeon, J. Phys. Chem. 98 (1994) 10189–10199.
[22] J.F. Huber, C.A. Meijers, J.A. Hulsman, Anal. Chem. 44 (1972) 111–116.
[23] T. Braumann, J. Chromatogr. 373 (1986) 191–225.
[24] C.A. Bergstrom, K. Luthman, P. Artursson, Eur. J. Pharm. Sci. 22 (2004) 387–398.
[25] T. Kuronita, E.L. Eskelinen, H. Fujita, P. Saftig, M. Himeno, Y. Tanaka, J. Cell. Sci. 115 (2002) 4117–4131.
[26] H.R. Ha, B. Stieger, G. Grassi, H.R. Altorfer, F. Follath, Eur. J. Clin. Pharmacol. 55 (2000) 807–814.
[27] H.R. Ha, L. Bigler, B. Wendt, M. Maggiorini, F. Follath, Eur. J. Pharm. Sci. 24 (2005) 271–279.
[28] A. Baritussio, S. Marzini, M. Agostini, A. Alberti, C. Cimenti, D. Bruttomesso, E. Manzato, D. Quaglino, A. Pettenazzo, Am. J. Physiol. Lung Cell. Mol. Physiol. 281 (2001) L1189–L1199.
[29] K. Eto, T. Yamashita, K. Hirose, Y. Tsubamoto, E.K. Ainscow, G.A. Rutter, S. Kimura, M. Noda, M. Iino, T. Kadowaki, Am. J. Physiol. Endocrinol. Metab. 285 (2003) E262–E271.