



# Itaconic acid hybrids as potential anticancer agents

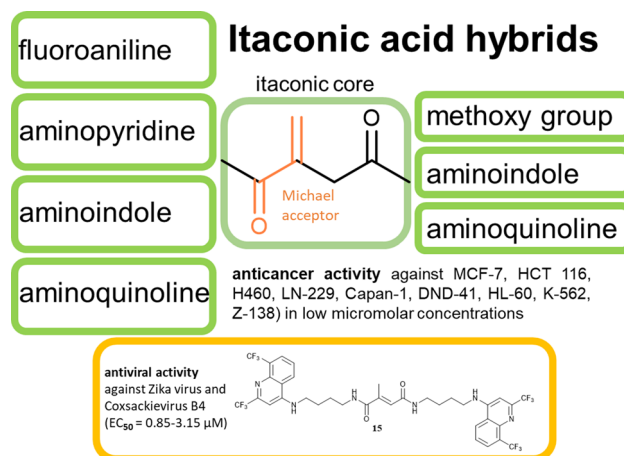
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## Abstract

In this paper, we report the synthesis of novel hybrids **2–14** based on itaconic acid and fluoroaniline, pyridine, indole and quinoline scaffolds. Itaconic acid is a naturally occurring compound with a Michael acceptor moiety, a key structural feature in several anticancer and antiviral drugs, responsible for the covalent binding of a drug to the cysteine residue of a specific protein. Aromatic parts of the hybrids also come from the substances reported as anticancer or antiviral agents. The synthetic route employed to access the amido-ester hybrids **2–13** used monomethyl itaconate or monomethyl itaconyl chloride and corresponding amines as the starting materials. Dimers **14** and **15** with two aminoindole or mefloquine moieties were prepared from itaconic acid and corresponding amino derivative, using standard coupling conditions (HATU/DIEA). All hybrids exerted anticancer effects in vitro against almost all the tumour cell lines that were evaluated (MCF-7, HCT 116, H460, LN-229, Capan-1, DND-41, HL-60, K-562, Z-138). Solid tumour cells were, in general, more responsive than the haematological cancer cells. The MCF-7 breast adenocarcinoma cell line appeared the most sensitive. Amido-ester **12** with chloroquine core and mefloquine homodimer **15** showed the highest activity with  $GI_{50}$  values between 0.7 and 8.6  $\mu$ M. In addition, compound **15** also exerted antiviral activity against Zika virus and Cocksackievirus B4 in low micromolar concentrations.

## Graphic abstract



**Keywords** Itaconic acid · Hybrid · Michael acceptor · Anticancer activity · Antiviral activity

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## Abbreviations

Capan-1	Human pancreatic adenocarcinoma cell line
CPE	Cytopathic effect
CQ	Chloroquine
DCC	<i>N,N'</i> -dicyclohexylcarbodiimide
DCM	Dichloromethane
DIEA	<i>N,N</i> -diisopropylethylamine

DND-41	T-acute lymphoblastic leukemia cell line
DTX	Docetaxel
FAN	Fluoroaniline
FBS	Foetal bovine serum
HATU	1-[bis(dimethylamino)methylene]-1 <i>H</i> -1,2,3-triazolo[4,5- <i>b</i> ]pyridinium 3-oxide hexafluorophosphate
H460	Lung carcinoma cell line
HBA	Number of H-bond acceptors
HBD	Number of H-bond donors
HCT 116	Colorectal carcinoma cell line
HL-60	Acute promyelocytic leukemia cell line
IA	Itaconic acid
IND	Indole
K-562	Chronic myelogenous leukemia cell line
LN-229	Human glioblastoma cell line
MCF-7	Breast adenocarcinoma cell line
MDCK	Madin-Darby Canine Kidney cell line
MR	Molar refractivity
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MQ	Mefloquine
MW	Microwave irradiation
PG	Percentage of growth
PSA	Molecular polar surface area
PQ	Primaquine
PYR	Pyridine
STS	Staurosporine
TEA	Trimethylamine
Z-138	Mantle cell lymphoma cell line

## Introduction

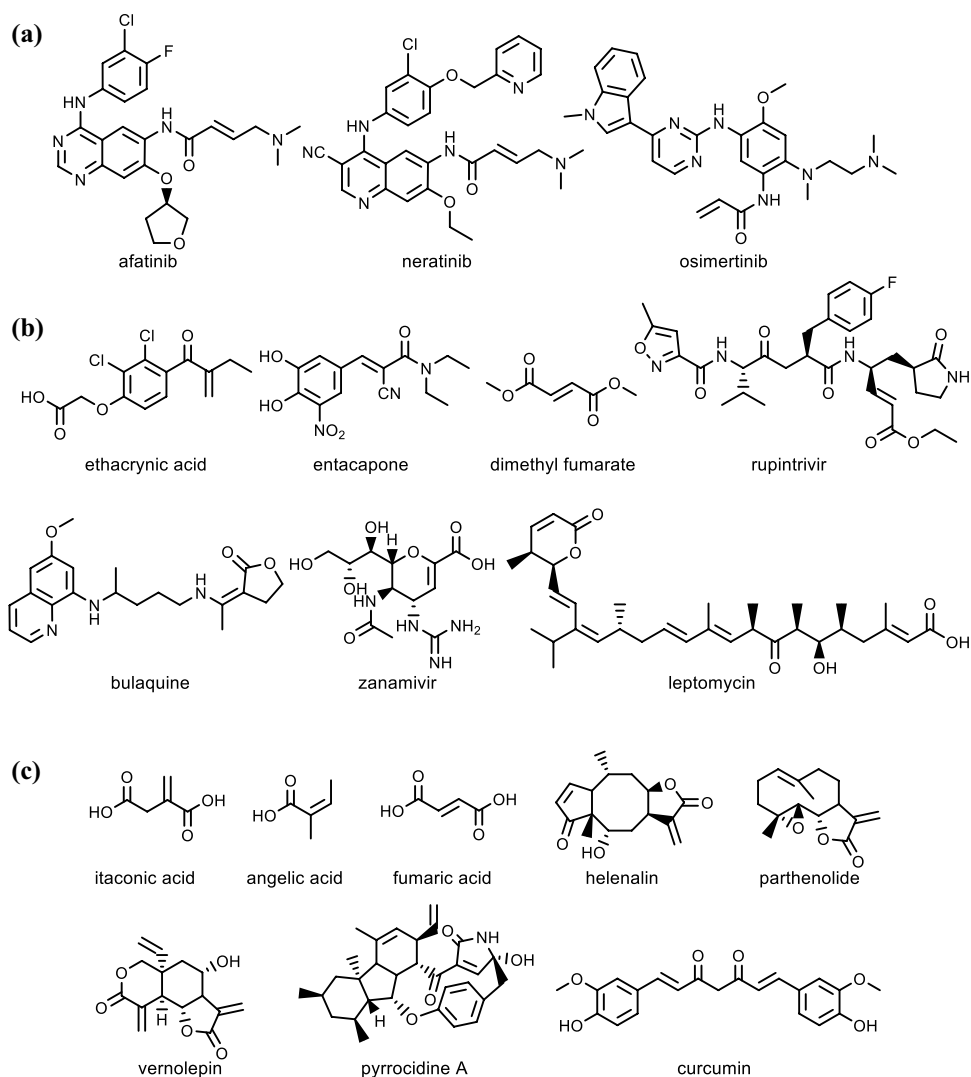
Itaconic acid (methylidenesuccinic acid, IA) is a dicarboxylic acid with exomethylene moiety conjugated with a carbonyl group. Compounds with itaconate core are naturally occurring substances isolated from various species of lichen and fungi. For example, chaetomelic acids, metabolic products of lichen *Chaetomella*, are known as strong farnesyltransferase inhibitors and valuable leads for the development of anticancer drugs [1, 2]. Itaconate is also a macrophage-specific metabolite—it mediates antimicrobial functions in the macrophage by inhibiting isocitrate lyase, an enzyme of the glyoxylate shunt part of a bacterial survival mechanism [3]. Weiss and collaborators showed that itaconic acid mediates crosstalk between macrophage metabolism and peritoneal tumours [4].

The reactive alpha-methylene unit in itaconic acid permits radical-mediated crosslinking or polymerization [5], so various polymers and copolymers derived from itaconic acid have been prepared [6, 7]. Compounds with the exposed unsaturated  $\beta$ -carbon atoms are considered as the

best substrates in conjugate addition (Michael addition). Michael acceptors are present in a number of drugs and also in many natural products (Fig. 1). Epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKI), like afatinib, neratinib, osimertinib, covalently bind to the cysteine residue located at the position 797 of the receptor [8–10], whereas ethacrynic acid with diuretic properties inhibits NKCC2 (Na–K-2Cl cotransporter) in the thick ascending loop of Henle and the macula densa. In addition, it inhibits the isoenzymes of glutathione S-transferase overexpressed in tumour tissues and reduces chemotherapy drug resistance [11]. Entacapone, dimethyl fumarate, rupintrivir, zanamivir and bulaquine are drugs used in the therapy of Parkinson's disease, psoriasis, multiple sclerosis, viral infections or malaria, respectively, whereas leptomycin has anticancer, antifungal and antibacterial activity [12]. Itaconic, angelic and fumaric acid, helenalin, parthenolide, vernoleptin, pyrrocidine A and curcumin are examples of numerous naturally occurring Michael acceptors.

To our surprise, itaconic acid derivatives are quite an underexplored area—only limited data of their preparation and/or biological activity are available in the literature [13, 14]. For example, itaconic acid diamides with anilines bearing F, Cl, Br, CF<sub>3</sub> or OCH<sub>3</sub> substituents at various positions are reported as anti-influenza agents that reduce virus replication by targeting virus nucleoproteins and disrupting virus ribonucleoprotein export from the nucleus to the cytosol [15]. On the other hand, aliphatic copolyester poly [butylene fumarate-co-butylene itaconate] obtained from itaconic acid, fumaric acid and 1,4-butanediol has shown low anticancer activity against MCF-7 adenocarcinoma cell line [16]. Since numerous primaquine (PQ) derivatives possess antiproliferative activity against the same cancer cell line [17–21], we decided to combine primaquine and itaconic structural motifs in single entities, so the goal of our research was to prepare IA-PQ hybrids and evaluate their anticancer potential, primarily against MCF-7 cell line. The analogue hybrids bearing similar quinoline antimalarial drugs, chloroquine (CQ) and mefloquine (MQ), were prepared as well. Namely, various antimalarials display either direct or adjuvant activity against cancer [22–24] and, as such, were evaluated or are currently under evaluation in numerous clinical trials against different cancer types, alone or in combination with conventional anticancer drugs/treatments [25]. In addition, we expanded our compound library with itaconic hybrids bearing other aromatic scaffolds, fluoroaniline (FAN), pyridine (PYR) or indole (IND), also present in anticancer agents (sorafenib, imatinib, sunitinib, tyrphostin AG 1112, to mention few of them). In this paper, we report the synthesis of fourteen novel IA-derivatives and evaluation of their anticancer and antiviral activity.

**Fig. 1** Chemical structures of compounds bearing  $\alpha,\beta$ -unsaturated carbonyl group: **a** anticancer drugs, **b** drugs from other therapeutic groups and **c** some naturally occurring substances



## Results and discussion

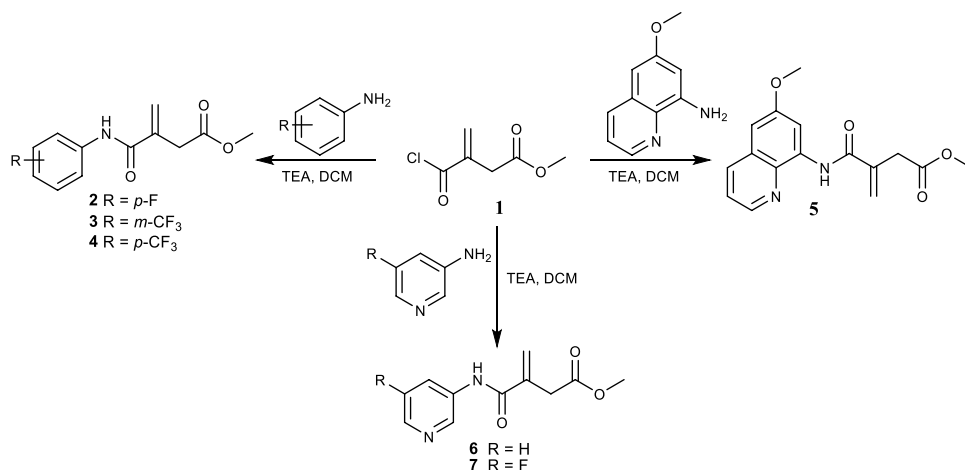
### Chemistry

Several sets of IA-hybrids were prepared: fluoroaniline derivatives **2–4** (IA-FAN) pyridine derivatives **6–9** (IA-PYR), indole derivatives **10** and **14** (IA-IND), PQ-derivatives **5** and **11** (IA-PQ), CQ-derivative **12** (IA-CQ) and two MQ-derivatives, amido-ester **13** and diamide **15** (IA-MQ). First, we had prepared IA-PQ conjugate **11** from primaquine and commercially available monomethyl itaconate. HATU was used as the coupling reagent, together with DIEA. The reaction proceeded smoothly and in good yields, in accord with previously reported similar reactions of primaquine with various dicarboxylic acids [21, 26]. Amido-esters **12** and **13** were obtained in analogous coupling reactions, starting from amines prepared from 1,4-butane diamine and 4,7-dichloro quinoline or 4-chloro-2,8-ditrifluoromethyl quinoline, respectively. However, coupling reactions with

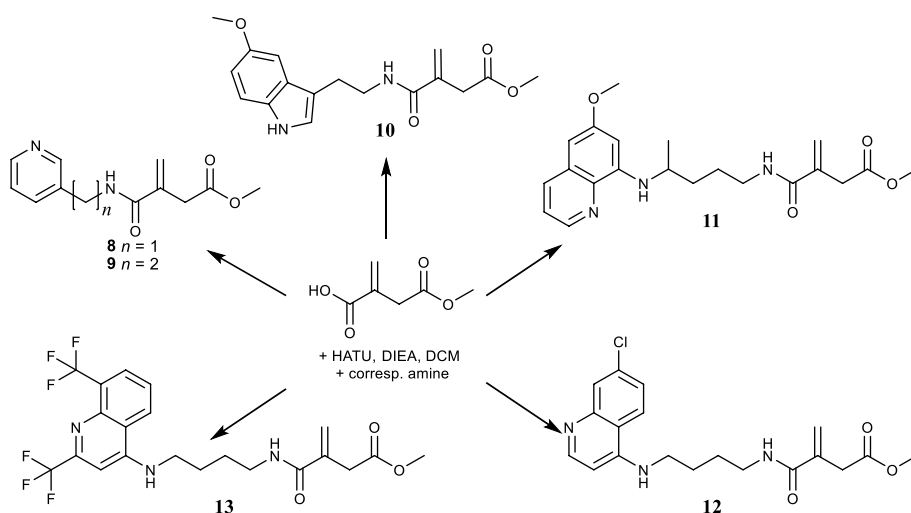
less basic amines, 8-amino-6-methoxyquinoline, 4-amino-7-chloroquinoline and 2,8-di-trifluoromethyl-4-aminoquinoline, failed in our hands. The substitution of HATU with *N,N'*-dicyclohexylcarbodiimide (DCC) was also unsuccessful. So, compound **5** was prepared by direct acylation of 8-amino-6-methoxyquinoline with chloride **1**, obtained from monomethyl itaconate and thionyl chloride. In this way, aniline derivatives **2–4** and two pyridine derivatives **6** and **7** were prepared as well, but 4-amino-7-chloroquinoline and 4-amino-2,8-ditrifluoromethylquinoline failed to react. The synthetic pathways leading to the IA-hybrids **2–13** are depicted in schemes 1 and 2. Finally, dimers **14** and **15** with two IND or MQ residues bridged by itaconic acid were prepared from itaconic acid and two equivalents of corresponding amine (Scheme 3). The amide bonds were achieved again by HATU/DIEA.

All new compounds were fully characterized by the usual spectroscopic methods (IR,  $^1\text{H}$ ,  $^{13}\text{C}$  NMR and MS) and elemental analyses. Spectral data are consistent with

**Scheme 1** Synthetic route for the preparation of IA-hybrids **2–7**



**Scheme 2** Synthetic route for the preparation of IA-hybrids **8–13**

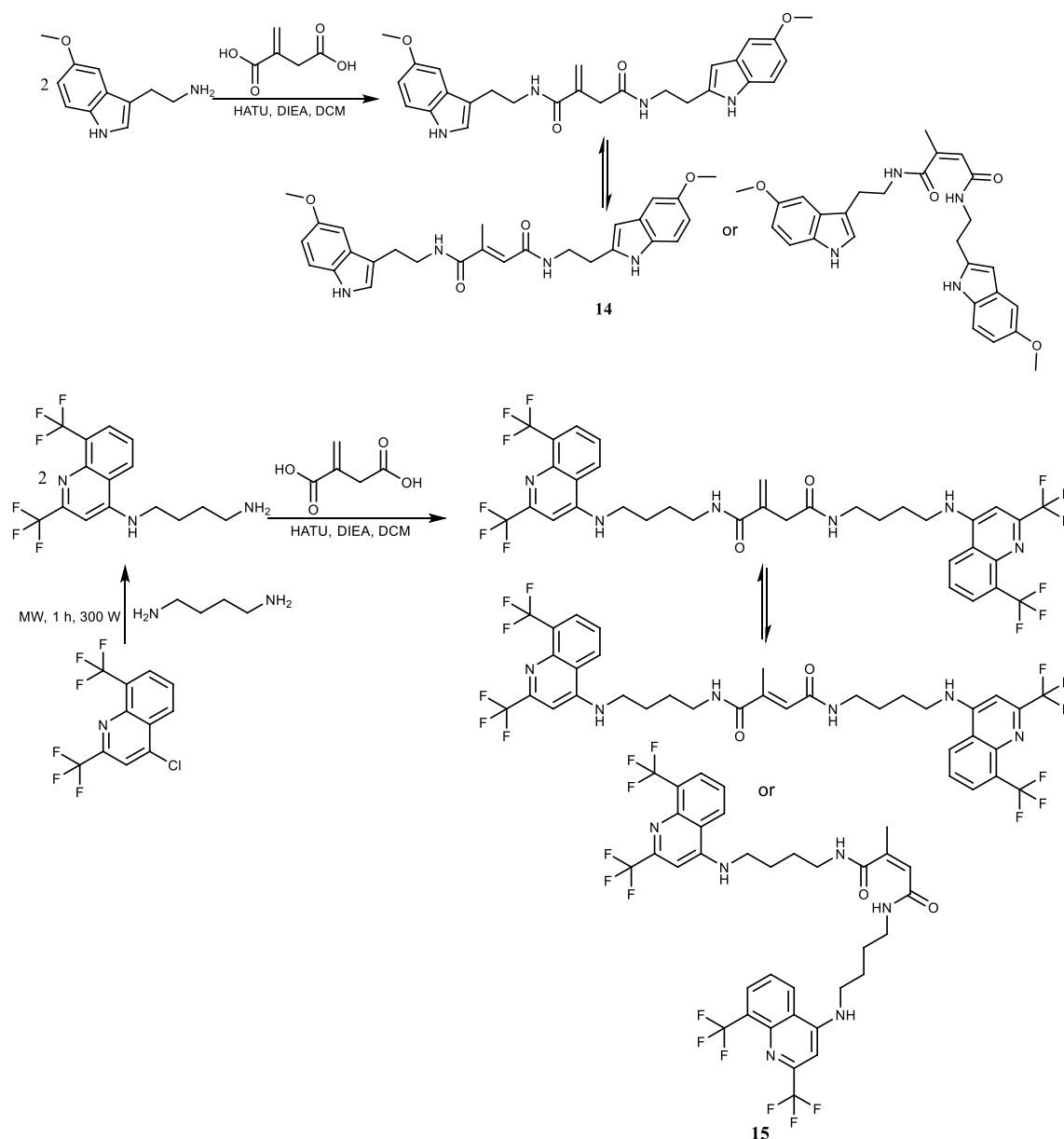


the proposed structures and are given in the Experimental section. In  $^{13}\text{C}$  NMR spectra, the amide carbonyl carbon appeared between  $\delta$  165 and 169 ppm, whereas the ester carbonyls in compounds **2–13** had signals approximately at 171 ppm. Exomethylene group gave two signals, first between 137.59 and 138.67 (the carbon atom in the chain) and the second between 121.01 and 124.01 (the exo-carbon atom). As was expected, the presence of trifluoromethyl groups in compounds **3**, **4**, **13** and **15** caused the splitting of signals into quartets (coupling constants approximately 271 Hz for CF<sub>3</sub> and 32 Hz for the adjacent carbons). Carbon atoms bearing fluorine in compounds **2** and **7** showed as doublets at approximately 158 ppm with  $J=239.8$  and 252.9 Hz, respectively. Coupling constants of the neighbouring carbon atoms were lower (the first neighbour 22–23 Hz and the second one 6–7 Hz). The signals of the quinoline scaffold gave the appropriate carbon atom counts in the aromatic region.

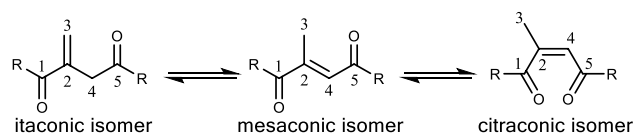
In  $^1\text{H}$  NMR spectra, amide NH signals of compounds **2–7** were visible as singlets between 10.06 and 10.48 ppm and

in compounds **8–15** as triplets between 7.96 and 9.10 ppm (all D<sub>2</sub>O exchangeable). Ester methyl group gave signals between 3.54 and 3.62 ppm. Methoxy group present in PQ-derivatives **5** and **11** was at a slightly higher value, at 3.83, while methoxy group in indole derivative **10** appeared at 3.76 ppm. Hydrogen atoms of the exomethylene group gave two singlets approximately at  $\delta$  6.3 and 5.8 ppm (compounds **2–7**) and 5.8 and 5.5 ppm (compounds **8–15**).

NMR spectra of compounds **14** and **15** confirmed the conversion of itaconic to mesaconic/citraconic isomers, according to Fig. 2 [27, 28]. In  $^{13}\text{C}$  NMR spectra signals of atoms C-2, C-3 and C-4 changed the position: C-2 shifted from 138 to 144, C-3 from 121 to 22, and C-4 from 37 to 121.  $^1\text{H}$  NMR spectrum also confirmed this isomerisation: two singlets of hydrogen atoms at C-3 shifted downfield to one singlet at 1.88 ppm (methyl), and signal of C-4 methylene group significantly shifted from 3.30 to 5.80 ppm and changed the integral value from two to one. However, configuration of the double bond in the isomerized products was not determined.



**Scheme 3** Synthetic route for the preparation of IND and MQ dimers **14** and **15**



**Fig. 2** The isomerisation of itaconic acid moiety

Further, we calculated the drug-like parameters for compounds **2–14** using the Chemicalize.org program [29]. The following parameters were determined and are outlined in Table 1: molecular weight (MW), partition coefficient (log *P*), number of H-bond donors (HBD), number of H-bond

acceptors (HBA), molecular polar surface area (PSA), molecular refractivity (MR) and polar surface area (PSA). The title compounds are fully in agreement with the Lipinski rule of five [30]. SwissADME Bioavailability Radars for selected hybrids are presented in Fig. 3 [31]. On the other hand, bis-derivative **15** is a huge molecule and, as such, is outside of the expected values for prospective small molecules. However, similar symmetric and asymmetric bis-quinoline or bis-artemisinin compounds are known for their antimalarial and/or anticancer activity [32–34] with one of them, piperazine, registered for malaria treatment as a single drug at first, and later in the combination with dihydroartemisinin [35].

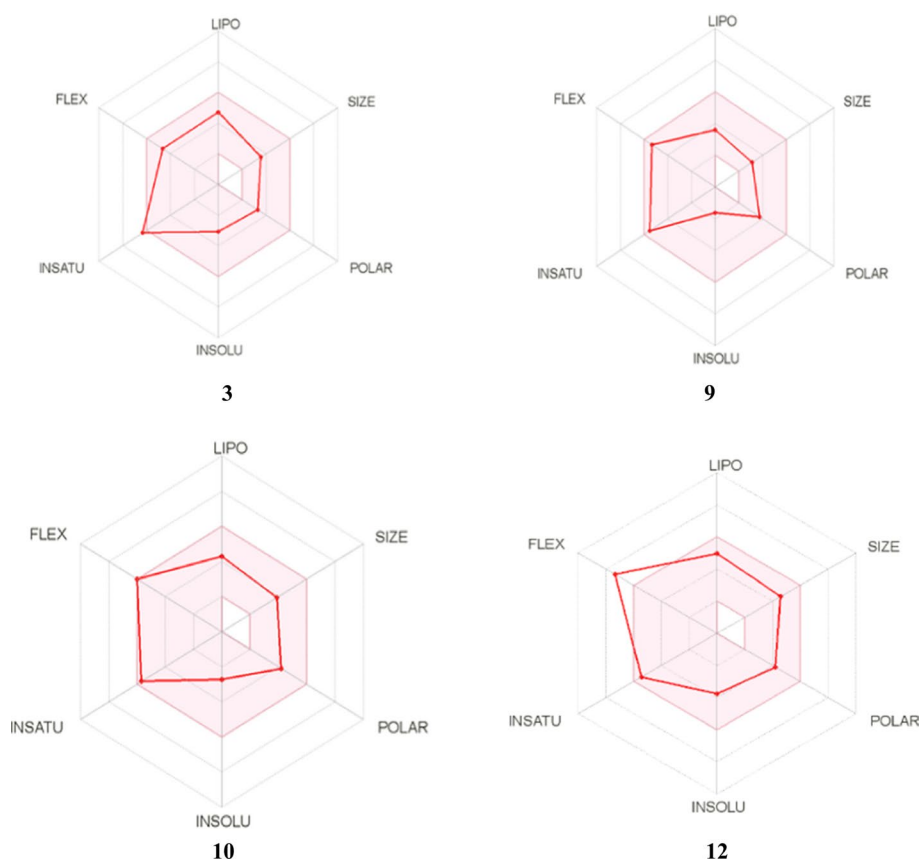
**Table 1** The Lipinski and Gelovani parameters of the itaconic-quinoline hybrids **2–14** calculated with Chemicalize.org program [29]

Compd.	Molecular formula	Number of atoms	MW	log <i>P</i>	HBD	HBA	Lipinski score <sup>a</sup>	MR (cm <sup>3</sup> /mol)	PSA (Å <sup>2</sup> )
<b>2</b>	C <sub>12</sub> H <sub>12</sub> FNO <sub>3</sub>	29	237.23	1.78	1	2	4	61.18	55.40
<b>3</b>	C <sub>13</sub> H <sub>12</sub> F <sub>3</sub> NO <sub>3</sub>	32	287.24	2.51	1	2	4	66.94	55.40
<b>4</b>	C <sub>13</sub> H <sub>12</sub> F <sub>3</sub> NO <sub>3</sub>	32	287.24	2.51	1	2	4	66.94	55.40
<b>5</b>	C <sub>16</sub> H <sub>16</sub> N <sub>2</sub> O <sub>4</sub>	38	300.31	1.63	1	4	4	81.35	77.52
<b>6</b>	C <sub>11</sub> H <sub>12</sub> N <sub>2</sub> O <sub>3</sub>	28	220.23	0.42	1	3	4	58.80	68.29
<b>7</b>	C <sub>11</sub> H <sub>11</sub> FN <sub>2</sub> O <sub>3</sub>	28	238.22	0.56	1	3	4	59.02	68.29
<b>8</b>	C <sub>12</sub> H <sub>14</sub> N <sub>2</sub> O <sub>3</sub>	31	234.26	0.12	1	3	4	61.86	68.29
<b>9</b>	C <sub>13</sub> H <sub>16</sub> N <sub>2</sub> O <sub>3</sub>	34	248.28	0.41	1	3	4	66.61	68.29
<b>10</b>	C <sub>17</sub> H <sub>20</sub> N <sub>2</sub> O <sub>4</sub>	43	316.36	1.57	2	3	4	86.32	80.42
<b>11</b>	C <sub>21</sub> H <sub>27</sub> N <sub>3</sub> O <sub>4</sub>	55	385.46	1.88	2	5	4	108.00	89.55
<b>12</b>	C <sub>19</sub> H <sub>22</sub> ClN <sub>3</sub> O <sub>3</sub>	48	375.85	2.23	2	4	4	101.92	80.32
<b>13</b>	C <sub>21</sub> H <sub>21</sub> F <sub>6</sub> N <sub>3</sub> O <sub>3</sub>	54	477.41	3.77	2	4	4	108.69	80.32
<b>14</b>	C <sub>27</sub> H <sub>30</sub> N <sub>4</sub> O <sub>4</sub>	65	474.56	2.79	4	4	4	136.22	108.24

MW, molecular weight; log *P*, partition coefficient; HBD, number of H-bond donors; HBA, number of H-bond acceptors; MR, molecular refractivity; PSA, polar surface area

<sup>a</sup>Out of four

**Fig. 3** Bioavailability Radars for selected IA-hybrids [31]. The pink area represents the optimal range for each of the properties (lipophilicity, size, polarity, solubility, saturation and flexibility)



## Anticancer activity

Anticancer activity of novel compounds based on itaconic acid and aromatic scaffolds was evaluated in vitro on five

cell lines of human solid tumours (MCF-7, breast adenocarcinoma; HCT 116, colorectal carcinoma; H460, lung carcinoma; LN-229, human glioblastoma; Capan-1, human pancreatic adenocarcinoma) and four haematological cancer cell

lines (DND-41, T-acute lymphoblastic leukemia; HL-60, acute promyelocytic leukemia; K-562, chronic myelogenous leukemia; Z-138 mantle cell lymphoma). Anticancer drug docetaxel (DTX) and a prototypical ATP-competitive kinase inhibitor staurosporine (STS) were used as reference compounds. All IA-hybrids were found to be highly effective against almost all of the cancer cell lines (Table 2). Solid tumour cells were, in general, more responsive to the treatment than the haematological cancer cells. MCF-7 was the most sensitive cell line: almost all  $GI_{50}$  values against MCF-7 were in one-digit micromolar range. On the other hand, K-562 was the least sensitive cell line, followed by DND-41. However, CQ-derivative **12** and MQ-homodimer **15** were effective against all evaluated cell lines, including these two, with  $GI_{50}$  values between 0.7 and 8.6  $\mu$ M. IA-FANs **2–4** exerted also high activity towards all cell lines and *m*-trifluoromethyl derivative **3** was the most effective between them. Indole derivative **10** was also very active, whereas MQ derivative **13** showed selectivity towards solid tumour cells. IA-PYR derivatives **6–9** were slightly less active than the other IA hybrids. The comparison of conjugates **5** and **11** with PQ-cores revealed that amido-ester **11** with the whole PQ-moiety was more active than derivative **5** without PQ-aliphatic chain and an additional nitrogen atom. Compounds **5** and **11** were more active against solid tumour cells than the parent drug, but not against haematological

cancer cells. Two MQ-derivatives, **13** and **15**, showed similar activity as mefloquine, although compound with two MQ-units was slightly superior. CQ-derivative **12** showed considerably better activity than chloroquine. Comparison of compounds **11**, **12** and **13**, which share the same central part and the ester functionality with difference in the quinoline part of the molecule, pointed the following order of activity: CQ-derivative > MQ-derivative > PQ-derivative.

High anticancer activities of all IA-hybrids support our findings on importance of double bond motif. In our previous papers, we have prepared primaquine and halogenaniline asymmetric fumardiamides and their reduced analogues, succindiamides (compounds bearing the same left- and right-wing, and the same four-carbon spacer, one series with and the second series without double bond in the central part) and evaluated their anticancer activity. Without exception, saturated analogues were always less active or completely inactive [26].

### Antiviral screening

Compounds **2–15** were evaluated against a broad variety of virus families including herpes simplex virus-1, herpes simplex virus-2, vaccinia virus, adenovirus-2, human coronavirus (229E), vesicular stomatitis virus, Cocksackievirus B4, respiratory syncytial virus, reovirus-1, sindbis virus,

**Table 2** Anticancer screening of IA-hybrids towards 9 different human tumour cell lines

Compd.	$GI_{50}$ ( $\mu$ M)								
	MCF-7	HCT 116	H460	LN-229	Capan-1	DND-41	HL-60	K-562	Z-138
<b>2</b>	2.0 $\pm$ 0.1	4.0 $\pm$ 1.0	15.0 $\pm$ 1.0	8.0 $\pm$ 0.6	2.8 $\pm$ 0.6	15.7 $\pm$ 0.0	9.3 $\pm$ 0.5	15.9 $\pm$ 1.0	8.4 $\pm$ 0.5
<b>3</b>	2.0 $\pm$ 0.4	3.0 $\pm$ 1.0	9.0 $\pm$ 2.0	4.4 $\pm$ 0.1	1.9 $\pm$ 0.3	21.2 $\pm$ 4.0	3.7 $\pm$ 0.1	13.9 $\pm$ 0.6	5.4 $\pm$ 0.1
<b>4</b>	1.0 $\pm$ 0.3	3.0 $\pm$ 1.0	11.0 $\pm$ 3.0	7.7 $\pm$ 0.2	5.3 $\pm$ 0.5	16.1 $\pm$ 0.4	8.0 $\pm$ 0.8	31.3 $\pm$ 3.0	8.2 $\pm$ 0.4
<b>5</b>	10.0 $\pm$ 2.5	12.0 $\pm$ 2.0	8.0 $\pm$ 2.0	6.2 $\pm$ 0.4	6.3 $\pm$ 2.3	25.5 $\pm$ 9.4	10.1 $\pm$ 0.1	31.0 $\pm$ 3.9	6.1 $\pm$ 0.3
<b>6</b>	3.0 $\pm$ 1.0	12.0 $\pm$ 2.0	18.0 $\pm$ 1.0	10.2 $\pm$ 1.0	8.9 $\pm$ 0.1	26.3 $\pm$ 6.6	13.1 $\pm$ 1.0	49.9 $\pm$ 2.2	10.9 $\pm$ 0.0
<b>7</b>	3.0 $\pm$ 1.1	7.0 $\pm$ 2.0	16.0 $\pm$ 2.0	8.1 $\pm$ 0.3	6.8 $\pm$ 1.7	21.0 $\pm$ 2.2	10.6 $\pm$ 0.7	43.4 $\pm$ 0.0	9.0 $\pm$ 0.2
<b>8</b>	6.0 $\pm$ 1.0	3.0 $\pm$ 1.0	16.0 $\pm$ 2.0	8.4 $\pm$ 0.1	9.1 $\pm$ 0.7	16.3 $\pm$ 0.1	11.0 $\pm$ 0.3	53.3 $\pm$ 2.7	10.2 $\pm$ 0.3
<b>9</b>	2.0 $\pm$ 1.0	4.0 $\pm$ 1.0	15.0 $\pm$ 1.0	6.2 $\pm$ 0.8	5.9 $\pm$ 0.2	18.5 $\pm$ 2.4	9.6 $\pm$ 0.6	34.5 $\pm$ 8.7	5.6 $\pm$ 1.6
<b>10</b>	2.0 $\pm$ 1.2	3.0 $\pm$ 1.0	11.0 $\pm$ 1.0	6.5 $\pm$ 1.2	2.4 $\pm$ 0.9	18.9 $\pm$ 4.2	4.9 $\pm$ 0.8	16.5 $\pm$ 1.0	2.6 $\pm$ 0.3
<b>11</b>	4.0 $\pm$ 1.0	2.5 $\pm$ 0.07	4.0 $\pm$ 0.5	4.3 $\pm$ 2.2	5.4 $\pm$ 3.3	18.1 $\pm$ 1.1	11.1 $\pm$ 0.6	28.1 $\pm$ 7.1	11.0 $\pm$ 0.2
<b>12</b>	0.8 $\pm$ 0.3	2.0 $\pm$ 0.05	2.0 $\pm$ 0.9	1.1 $\pm$ 0.1	1.3 $\pm$ 0.4	3.1 $\pm$ 0.1	1.9 $\pm$ 0.0	8.1 $\pm$ 0.4	1.9 $\pm$ 0.1
<b>13</b>	1.4 $\pm$ 0.1	2.0 $\pm$ 0.8	3.5 $\pm$ 1.3	10.8 $\pm$ 2.5	32.2 $\pm$ 2.7	36.7 $\pm$ 10.0	10.3 $\pm$ 1.5	46.9 $\pm$ 0.7	12.2 $\pm$ 0.1
<b>14</b>	11.0 $\pm$ 0.3	13.0 $\pm$ 0.2	23.0 $\pm$ 1.0	nd <sup>a</sup>	nd	nd	nd	nd	nd
<b>15</b>	0.7 $\pm$ 0.2	2.4 $\pm$ 0.4	3.0 $\pm$ 1.0	2.4 $\pm$ 0.8	2.3 $\pm$ 0.3	7.9 $\pm$ 4.4	2.6 $\pm$ 0.2	8.6 $\pm$ 0.3	6.9 $\pm$ 1.3
PQ <sup>b</sup>	7.0 $\pm$ 1.5	17.0 $\pm$ 1.0	17.0 $\pm$ 3.0	9.9 $\pm$ 0.4	9.0 $\pm$ 3.2	14.0 $\pm$ 1.0	7.0 $\pm$ 0.5	43.3 $\pm$ 0.1	10.2 $\pm$ 0.5
CQ <sup>c</sup>	6.0 $\pm$ 1.8	16.0 $\pm$ 3.0	13.0 $\pm$ 0.8	22.0 $\pm$ 2.8	14.9 $\pm$ 1.5	53.7 $\pm$ 6.9	13.0 $\pm$ 0.2	45.0 $\pm$ 0.7	13.6 $\pm$ 0.6
MQ <sup>d</sup>	1.3 $\pm$ 0.2	2.0 $\pm$ 0.1	1.5 $\pm$ 0.1	0.1 $\pm$ 0.1	2.0 $\pm$ 0.1	3.2 $\pm$ 0.2	5.9 $\pm$ 3.2	9.4 $\pm$ 0.3	12.6 $\pm$ 0.1
DTX <sup>e,*</sup>	nd	4.5 $\pm$ 4.4	5.8 $\pm$ 3.8	3.5 $\pm$ 1.2	4.5 $\pm$ 2.0	2.0 $\pm$ 1.2	10.4 $\pm$ 2.4	9.6 $\pm$ 3.3	1.4 $\pm$ 0.9
STS <sup>f,*</sup>	nd	23.0 $\pm$ 11.4	48.0 $\pm$ 2.3	63.4 $\pm$ 22.3	32.3 $\pm$ 3.2	16.1 $\pm$ 10.5	31.2 $\pm$ 12.3	33.7 $\pm$ 3.6	26.1 $\pm$ 13.9

<sup>a</sup>nd, not determined; <sup>b</sup>primaquine diphosphate; <sup>c</sup>CQ, chloroquine diphosphate; <sup>d</sup>MQ, mefloquine hydrochloride; <sup>e</sup>DTX, docetaxel; <sup>f</sup>STS, staurosporine. \*Concentrations in nM. Two individual experiments were carried out and each tested concentration was performed at least in triplicate



Punta Toro virus, yellow fever virus, Zika virus, influenza A/H1N1 (A/Ned/378/05), influenza A/H3N2 (A/HK/7/87) and influenza B (B/Ned/537/05). The activities of the compounds were compared with the activities of the parent drugs (PQ, CQ and MQ), DS-10.000 and standard antiviral drugs (ribavirin, mycophenolic acid, brivudin, cidofovir, aciclovir, ganciclovir, zalcitabine, alovudine, UDA, zanamivir or rimantadine, depending on the virus type). Almost all compounds were inactive ( $EC_{50} > 100 \mu\text{mol L}^{-1}$ ), with the exception of MQ-homodimer **15**. This compound exerted antiviral activity against Zika virus and Cocksackievirus B4.  $EC_{50}$  against Zika virus was  $0.85 \mu\text{mol L}^{-1}$  when measuring the cell viability with the colorimetric formazan-based MTS assay, while the  $EC_{50}$  as determined by visual scoring was  $2.35 \mu\text{mol L}^{-1}$  ( $EC_{50}$  values for DS-10.000 were 2.9 and  $4 \mu\text{mol L}^{-1}$ , whereas for mycophenolic acid were 1.2 and  $1.8 \mu\text{mol L}^{-1}$ ). Comparable antiviral activity was obtained against Cocksackievirus B4 with  $EC_{50}$  values 1.5 and  $3.15 \mu\text{mol L}^{-1}$ , respectively.  $EC_{50}$  values for DS-10.000 were 5.8 and  $10 \mu\text{mol L}^{-1}$ , and for ribavirin much higher ( $109$  and  $183 \mu\text{mol L}^{-1}$ ). The  $CC_{50}$  values of compound **15** were between  $6.7$  and  $10 \mu\text{mol L}^{-1}$  in Vero or Hep-2 cells in which the replication of Zika virus or Cocksackievirus B4 was measured.

### Interaction with glutathione (GSH)

In the drug development process, it is usual to evaluate the Michael acceptors interaction with glutathione (GSH), cysteine containing tripeptide, which plays important roles in antioxidant defence, nutrient metabolism and regulation of cellular events [36]. Two the most active compounds, namely compound **2** and **12**, were chosen for the testing. Incubation of  $1 \mu\text{M}$  solutions of compounds **2** and **12** with GSH ( $125 \mu\text{M}$ ) during 6 days revealed that our compounds do not affect the maintenance of GSH homeostasis. This is important finding, since the GSH depleting compounds switch the redox state towards an oxidant condition provoking oxidative/nitrosative stress and inflammation, which lead to apoptosis and/or autophagy of the enterocytes [37].

### Conclusions

Itaconic acid-based hybrids with fluoroaniline, pyridine, indole and quinoline scaffolds were designed as potential antiproliferative and/or antiviral agents and prepared in a simple, clean and inexpensive way. In vitro evaluation against nine cancer cell lines confirmed their anticancer activity mostly in low micromolar concentrations. IA-quinoline hybrids **12** and **15** were the most active compounds. Compound **15** exerted also antiviral activity against Zika virus and Cocksackievirus B4 replication. With their high

activity and favourable drug-like properties itaconic-hybrids could serve as starting compounds for further optimization and development of more potent anticancer agents.

## Experimental

### Materials and methods

Melting points were measured on Stuart Melting Point (SMP3) apparatus (Barloworld Scientific, UK) in open capillaries with uncorrected values. IR spectra were recorded on FTIR Perkin Elmer Paragon 500. All NMR ( $^1\text{H}$  and  $^{13}\text{C}$ ) spectra were recorded in DMSO- $d_6$  at  $25^\circ\text{C}$  on NMR Avance 400 (Bruker, Germany at  $400 \text{ MHz}$  for  $^1\text{H}$  and  $101 \text{ MHz}$  for  $^{13}\text{C}$  nuclei, respectively). Chemical shifts ( $\delta$ ) are reported in parts per million (ppm) using tetramethylsilane as a reference in the  $^1\text{H}$  and the DMSO residual peak as a reference in the  $^{13}\text{C}$  spectra ( $39.52 \text{ ppm}$ ). Coupling constants ( $J$ ) are reported in hertz (Hz). Mass spectra were recorded on HPLC-MS/MS (HPLC, Agilent Technologies 1200 Series; MS, Agilent Technologies 6410 Triple Quad) and Synapt G2-Si ESI-QTOF-MS system (Waters). The mass determination was realized using electron spray ionization (ESI) in positive mode. Elemental analyses were performed on the CHNS LECO analyzer (LECO Corporation, USA). All compounds were routinely checked by thin-layer chromatography with Merck silica gel 60F-254 glass plates using the following solvent systems: cyclohexane/ethyl acetate/methanol 10:10:5, dichloromethane/methanol 98:2, 95:5 and 9:1. Spots were visualized by short-wave UV light and iodine vapor. Column chromatography was performed on silica gel  $60 \text{ \AA}$ ,  $70\text{--}230 \text{ mesh}$  (Sigma-Aldrich, USA) with the same eluents used in TLC. All chemicals, solvents and biochemical reagents were of analytical grade and purchased from commercial sources. DIEA was purchased from ThermoFisher (Germany). Monomethyl itaconate, HATU, 5-methoxytryptamine, 3-aminopyridine, 3-amino-5-fluoropyridine, 3-picolylamine and 3-(2-aminoethyl)pyridine were purchased from TCI chemicals (Japan). Triethylamine (TEA), 4,7-dichloroquinoline, 4-chloro-2,8-bis(trifluoromethyl)quinoline, 8-amino-6-methoxyquinoline hydrobromide, 4-amino-7-chloroquinoline, 4-amino-2,8-bis(trifluoromethyl)quinoline and PQ bisphosphate were purchased from Sigma-Aldrich (USA). PQ base and 8-amino-6-methoxyquinoline were prepared prior to the use from PQ bisphosphate and 8-amino-6-methoxyquinoline hydrobromide, respectively. All reactions with PQ were run light protected.  $N^1$ -(7-chloroquinolin-4-yl)butane-1,4-diamine and  $N^1$ -[2,8-bis(trifluoromethyl)quinolin-4-yl]butane-1,4-diamine were prepared from 1,4-diaminobutane and 4,7-dichloroquinoline or 4-chloro-2,8-bis(trifluoromethyl)



quinoline under microwave irradiation (300 W) at 95 °C, 30 or 60 min [38, 39].

## General procedure for the synthesis of amido-esters 2–13

Compounds **2–13** were prepared from monomethyl itaconyl chloride **1** and corresponding amine (method A) or by condensation of the corresponding amine with monomethyl itaconate (method B), following the previously described procedures [21].

**Method A** A solution of monomethyl itaconate (0.072 g, 0.5 mmol), thionyl chloride (0.18 mL, 2.5 mmol) and two drops of dry dimethylformamide (DMF) in dry toluene was stirred for 0.5 h and concentrated under reduced pressure. The residue was triturated with toluene (2 × 10 mL), and the solvent was evaporated again to obtain crude chloride **1**. A solution of the corresponding amine (0.55 mmol) and TEA (0.5 mmol) in dry dichloromethane (5 mL) was added dropwise to the solution of **1** (0.081 g, 0.5 mmol) in dry dichloromethane (5 mL). The mixture was stirred at room temperature for 1 h and washed with brine (2 × 20 mL). The organic layer was dried with anhydrous sodium sulphate, filtered and evaporated under reduced pressure.

**Method B** A solution of monomethyl itaconate (0.072 g, 0.5 mmol), HATU (0.190 g, 0.5 mmol) and DIEA (0.129 g, 1 mmol) in dry dichloromethane (5 mL) was stirred at room temperature. After 10 min, a solution of the corresponding quinoline base (0.55 mmol) in dichloromethane (5 mL) was added. The mixture was stirred at room temperature for 0.5 h or overnight and was then evaporated under reduced pressure. The residue was dissolved in ethyl acetate (20 mL) and washed with brine (3 × 20 mL). The organic layer was dried over anhydrous sodium sulphate, filtered and evaporated under reduced pressure.

**Methyl 3-[(4-fluorophenyl)carbamoyl]but-3-enoate (2)** **2** was obtained by method A, from the reaction of chloride **1** (0.081 g, 0.5 mmol) and 4-fluoroaniline (0.061 g, 0.55 mmol) in the presence of TEA (0.051 g, 0.07 mL, 0.5 mmol) and after purification by column chromatography (dichloromethane/methanol = 98:2) as a pale oil (0.049 g, yield 41%); IR (film) ( $\nu_{\max}$ /cm<sup>-1</sup>): 3316, 3150, 3072, 2960, 1890, 1739, 1660, 1626, 1544, 1508, 1436, 1408, 1326, 1214, 1150, 1098, 1010, 934, 834, 780, 746, 674, 626; <sup>1</sup>H NMR ( $\delta$ /ppm): 10.06 (s, 1H), 7.70–7.67 (m, 2H), 7.17–7.13 (m, 2H), 6.03 (s, 1H), 5.72 (s, 1H), 3.59 (s, 3H), 3.41 (s, 2H); <sup>13</sup>C NMR ( $\delta$ /ppm): 171.08, 165.61, 158.15 (d,  $J$  = 239.8 Hz), 138.10, 135.37 (d,  $J$  = 2.6 Hz), 122.83, 121.77 (d,  $J$  = 7.7 Hz), 115.14 (d,  $J$  = 22.1 Hz), 51.59, 37.46; ESI-MS  $m/z$  = 260.1 (M + 23)<sup>+</sup>; Anal. Calcd. for C<sub>12</sub>H<sub>12</sub>FNO<sub>3</sub>: C, 60.76; H, 5.10; N, 5.90, found: C, 60.63; H, 5.13; N, 5.85.

**Methyl 3-[[3-(trifluoromethyl)phenyl]carbamoyl]but-3-enoate (3)** **3** was obtained by method A, from the reaction of chloride **1** (0.081 g, 0.5 mmol) and 3-(trifluoromethyl)aniline (0.089 g, 0.55 mmol) in the presence of TEA (0.051 g, 0.07 mL, 0.5 mmol) and after purification by column chromatography (dichloromethane/methanol = 98:2) and crystallization from petrolether/diethyl ether as a pale solid (0.052 g, yield 36%); mp 61–62 °C; IR (KBr) ( $\nu_{\max}$ /cm<sup>-1</sup>): 3380, 3118, 2960, 1718, 1684, 1638, 1566, 1496, 1442, 1394, 1340, 1216, 1170, 1114, 1068, 1018, 950, 892, 812, 746, 702, 628; <sup>1</sup>H NMR ( $\delta$ /ppm): 10.32 (s, 1H), 8.15 (s, 1H), 7.94 (d,  $J$  = 8.6 Hz, 1H), 7.56 (t,  $J$  = 7.7 Hz, 1H), 7.42 (d,  $J$  = 7.8 Hz, 1H), 6.09 (s, 1H), 5.78 (s, 1H), 3.60 (s, 3H), 3.44 (s, 2H); <sup>13</sup>C NMR ( $\delta$ /ppm): 171.04, 166.15, 139.80, 137.84, 129.85, 129.85–128.86 (q,  $J$  = 31.5 Hz), 123.55, 128.20–120.08 (q,  $J$  = 272.2 Hz), 123.42, 119.76, 115.95, 51.62, 37.37; ESI-MS  $m/z$  = 310.1 (M + 23)<sup>+</sup>; Anal. Calcd. for C<sub>13</sub>H<sub>12</sub>F<sub>3</sub>NO<sub>3</sub>: C, 54.36; H, 4.21; N, 4.88, found: C, 54.03; H, 4.38; N, 4.77.

**Methyl 3-[[4-(trifluoromethyl)phenyl]carbamoyl]but-3-enoate (4)** **4** was obtained by method A, from the reaction of chloride **1** (0.081 g, 0.5 mmol) and 3-(trifluoromethyl)aniline (0.089 g, 0.55 mmol) in the presence of TEA (0.051 g, 0.07 mL, 0.5 mmol) and after purification by column chromatography (dichloromethane/methanol = 98:2) and trituration with petrolether as a pale solid (0.055 g, yield 38%); mp 101–103 °C; IR (KBr) ( $\nu_{\max}$ /cm<sup>-1</sup>): 3372, 3204, 3068, 3008, 2960, 1722, 1686, 1636, 1604, 1548, 1444, 1412, 1330, 1254, 1214, 1166, 1112, 1066, 1014, 942, 902, 854, 800, 744; <sup>1</sup>H NMR ( $\delta$ /ppm): 10.35 (s, 1H), 7.91 (d,  $J$  = 8.2 Hz, 2H), 7.69 (d,  $J$  = 8.1 Hz, 2H), 6.09 (s, 1H), 5.79 (s, 1H), 3.60 (s, 3H), 3.44 (s, 2H); <sup>13</sup>C NMR ( $\delta$ /ppm): 171.04, 166.21, 142.66, 137.86, 128.34–120.39 (q,  $J$  = 271.3 Hz), 125.91, 123.67, 122.98–123.93 (q,  $J$  = 31.9 Hz), 119.77, 51.62, 37.39; ESI-MS  $m/z$  = 310.2 (M + 23)<sup>+</sup>; Anal. Calcd. for C<sub>13</sub>H<sub>12</sub>F<sub>3</sub>NO<sub>3</sub>: C, 54.36; H, 4.21; N, 4.88, found: C, 54.44; H, 4.24; N, 4.63.

**Methyl 3-[(6-methoxyquinolin-8-yl)carbamoyl]but-3-enoate (5)** **5** was obtained by method A, from the reaction of chloride **1** (0.081 g, 0.5 mmol) and 8-amino-6-methoxyquinoline, obtained from 8-amino-6-methoxyquinoline hydrobromide (0.140 g, 0.55 mol) and TEA (0.090 g, 0.12 mL, 0.89 mmol) in dichloromethane (5 mL) and after purification by column chromatography (dichloromethane/methanol = 98:2) and recrystallization from diethyl ether/petrolether as a pale solid (0.033 g, yield 29%); mp 94–95 °C; IR (KBr) ( $\nu_{\max}$ /cm<sup>-1</sup>): 3334, 1738, 1676, 1634, 1596, 1536, 1458, 1426, 1390, 1336, 1218, 1162, 1026, 934, 876, 834, 790, 750, 686; <sup>1</sup>H NMR ( $\delta$ /ppm): 10.27 (s, 1H), 8.76 (dd,  $J$  = 4.2, 1.6 Hz, 1H), 8.32 (dd,  $J$  = 8.3, 1.6 Hz, 1H), 8.25 (d,  $J$  = 2.7 Hz, 1H), 7.61 (dd,  $J$  = 8.3, 4.2 Hz, 1H), 7.12 (d,  $J$  = 2.7 Hz, 1H), 6.13 (s, 1H), 5.85 (d,  $J$  = 1.2 Hz, 1H), 3.90 (s, 3H), 3.62 (s, 3H), 3.53 (d,  $J$  = 1.0 Hz, 2H); <sup>13</sup>C NMR

( $\delta$ /ppm): 170.90, 165.13, 157.45, 146.40, 138.67, 135.50, 134.62, 134.43, 128.88, 122.95, 122.78, 108.50, 100.11, 55.57, 51.72, 37.17; ESI-MS  $m/z$  = 301.1 ( $M+1$ )<sup>+</sup>, 323.1 ( $M+23$ )<sup>+</sup>; Anal. Calcd. for C<sub>16</sub>H<sub>16</sub>N<sub>2</sub>O<sub>4</sub>: C, 63.99; H, 5.37; N, 9.33, found: C, 64.03; H, 5.34; N, 9.26.

**Methyl 3-[(pyridin-3-yl)carbamoyl]but-3-enoate (6)** **6** was obtained by method A, from the reaction of chloride **1** (0.081 g, 0.5 mmol) and 3-aminopyridine (0.052 g, 0.55 mmol) in the presence of TEA (0.051 g, 0.07 mL, 0.5 mmol) and after purification by column chromatography (dichloromethane/methanol = 95:5) as a dark oil (0.029 g, yield 26%); IR (film) ( $\nu_{\max}$ /cm<sup>-1</sup>): 3340, 3180, 3116, 3038, 2998, 2954, 2924, 2850, 1730, 1676, 1632, 1590, 1536, 1482, 1426, 1332, 1270, 1198, 1016, 942, 906, 848, 806, 748, 708, 628; <sup>1</sup>H NMR ( $\delta$ /ppm): 10.22 (s, 1H), 8.83 (d,  $J$  = 2.6 Hz, 1H), 8.28 (dd,  $J$  = 4.7, 1.5 Hz, 1H), 8.10–8.07 (m, 1H), 7.37–7.34 (m, 1H), 6.08 (s, 1H), 5.78 (s, 1H), 3.59 (s, 3H), 3.43 (s, 2H); <sup>13</sup>C NMR ( $\delta$ /ppm): 171.04, 166.14, 144.42, 141.61, 137.80, 135.66, 126.89, 123.50, 51.63, 37.38; ESI-MS  $m/z$  = 221.1 ( $M+1$ )<sup>+</sup>; Anal. Calcd. for C<sub>11</sub>H<sub>12</sub>N<sub>2</sub>O<sub>3</sub>: C, 59.99; H, 5.49; N, 12.72, found: C, 59.71; H, 5.40; N, 12.39.

**Methyl 3-[(5-fluoropyridin-3-yl)carbamoyl]but-3-enoate (7)** **7** was obtained by method A, from the reaction of chloride **1** (0.081 g, 0.5 mmol) and 3-amino-5-fluoropyridine (0.062 g, 0.55 mmol) in the presence of TEA (0.051 g, 0.07 mL, 0.5 mmol) and after purification by column chromatography (dichloromethane/methanol = 9:1) as a pale solid (0.045 g, yield 38%); mp 82–84 °C; IR (KBr) ( $\nu_{\max}$ /cm<sup>-1</sup>): 3332, 3104, 2956, 2850, 1738, 1682, 1634, 1596, 1540, 1470, 1422, 1350, 1290, 1202, 1168, 1018, 942, 872, 802, 698; <sup>1</sup>H NMR ( $\delta$ /ppm): 10.48 (s, 1H), 8.70 (t,  $J$  = 1.8 Hz, 1H), 8.30 (d,  $J$  = 2.7 Hz, 1H), 8.11–8.07 (m, 1H), 6.10 (s, 1H), 5.82 (s, 1H), 3.60 (s, 3H), 3.45 (s, 2H); <sup>13</sup>C NMR ( $\delta$ /ppm): 170.98, 166.50, 158.61 (d,  $J$  = 252.9 Hz), 137.57, 137.53, 136.93 (d,  $J$  = 6.2 Hz), 131.93 (d,  $J$  = 22.6 Hz), 124.07, 113.48 (d,  $J$  = 22.5 Hz), 51.66, 37.28; ESI-MS  $m/z$  = 239.2 ( $M+1$ )<sup>+</sup>; Anal. Calcd. for C<sub>11</sub>H<sub>11</sub>FN<sub>2</sub>O<sub>3</sub>: C, 55.46; H, 4.65; N, 11.76, found: C, 55.09; H, 4.67; N, 11.93.

**Methyl 3-[(pyridin-3-yl)methyl]carbamoyl]but-3-enoate (8)** **8** was obtained by method B, from the reaction of monomethyl itaconate (0.072 g, 0.5 mmol), HATU (0.190 g, 0.5 mmol), DIEA (0.129 g, 1 mmol) and 3-picolylamine (0.059 g, 0.06 mL, 0.55 mmol) and after purification by column chromatography (dichloromethane/methanol = 95:5) as a pale oil (0.057 g, yield 49%); IR (film) ( $\nu_{\max}$ /cm<sup>-1</sup>): 3318, 3030, 2918, 2850, 1732, 1664, 1538, 1430, 1320, 1262, 1148, 1010, 934, 838, 708; <sup>1</sup>H NMR ( $\delta$ /ppm): 8.74 (t,  $J$  = 6.0 Hz, 1H), 8.48 (d,  $J$  = 2.2 Hz, 1H), 8.44 (dd,  $J$  = 4.9, 1.7 Hz, 1H), 7.64 (dt,  $J$  = 7.8, 2.0 Hz, 1H), 7.36–7.33 (m, 1H), 5.89 (s, 1H), 5.57 (s, 1H), 4.34 (d,  $J$  = 6.0 Hz, 2H), 3.56 (s, 3H), 3.32 (s, 2H); <sup>13</sup>C NMR ( $\delta$ /ppm): 171.01, 166.77,

148.62, 148.00, 137.78, 135.03, 134.85, 123.39, 121.82, 51.49, 40.01, 37.52; ESI-MS  $m/z$  = 235.2 ( $M+1$ )<sup>+</sup>; Anal. Calcd. for C<sub>12</sub>H<sub>14</sub>N<sub>2</sub>O<sub>3</sub>: C, 61.53; H, 6.02; N, 11.96, found: C, 61.59; H, 6.25; N, 11.80.

**Methyl 3-[[2-(pyridin-3-yl)ethyl]carbamoyl]but-3-enoate (9)** **9** was obtained by method A, from the reaction of chloride **1** (0.081 g, 0.5 mmol) and 3-(2-aminoethyl)pyridine (0.067 g, 0.07 mL, 0.55 mmol) in the presence of TEA (0.051 g, 0.07 mL, 0.5 mmol) and after purification by column chromatography (dichloromethane/methanol = 9:1) as a pale oil (0.011 g, yield 9%); IR (film) ( $\nu_{\max}$ /cm<sup>-1</sup>): 3318, 3034, 2998, 2952, 2858, 1738, 1662, 1620, 1538, 1480, 1430, 1322, 1264, 1200, 1168, 1020, 938, 876, 840, 800, 748, 716; <sup>1</sup>H NMR ( $\delta$ /ppm): 8.42–8.40 (m, 2H), 8.23 (t,  $J$  = 5.7 Hz, 1H), 7.62 (dt,  $J$  = 7.8, 1.9 Hz, 1H), 7.33–7.291 (m, 1H), 5.78 (s, 1H), 5.50 (s, 1H), 3.57 (s, 3H), 3.36–3.32 (q,  $J$  = 7.0 Hz, 2H), 3.29 (s, 2H), 2.77 (t,  $J$  = 7.1 Hz, 2H); <sup>13</sup>C NMR ( $\delta$ /ppm): 171.01, 166.57, 149.87, 147.38, 137.96, 136.23, 135.00, 123.40, 121.33, 51.48, 40.09, 37.46, 31.94; ESI-MS  $m/z$  = 249.2 ( $M+1$ )<sup>+</sup>; Anal. Calcd. for C<sub>13</sub>H<sub>16</sub>N<sub>2</sub>O<sub>3</sub>: C, 62.89; H, 6.50; N, 11.28, found: C, 62.94; H, 6.57; N, 11.51.

**Methyl 3-[[2-(5-methoxy-1H-indol-3-yl)ethyl]carbamoyl]but-3-enoate (10)** **10** was obtained by method B, from the reaction of monomethyl itaconate (0.072 g, 0.5 mmol), HATU (0.190 g, 0.5 mmol), DIEA (0.129 g, 1 mmol) and 5-methoxytryptamine (0.105 g, 0.55 mmol) and after purification by column chromatography (dichloromethane/methanol = 9:1) as a pale oil (0.085 g, yield 54%); IR (film) ( $\nu_{\max}$ /cm<sup>-1</sup>): 3322, 3054, 2948, 1738, 1660, 1620, 1536, 1486, 1438, 1322, 1264, 1216, 1172, 1072, 1028, 926, 800, 736, 702; <sup>1</sup>H NMR ( $\delta$ /ppm): 10.64 (s, 1H), 8.24 (t,  $J$  = 5.7 Hz, 1H), 7.22 (d,  $J$  = 8.8 Hz, 1H), 7.10 (d,  $J$  = 2.4 Hz, 1H), 7.03 (d,  $J$  = 2.4 Hz, 1H), 6.71 (dd,  $J$  = 8.8, 2.4 Hz, 1H), 5.83 (s, 1H), 5.51 (s, 1H), 3.76 (s, 3H), 3.57 (s, 3H), 3.39–3.34 (m, 2H), 3.31 (s, 2H), 2.82 (t,  $J$  = 7.5 Hz, 2H); <sup>13</sup>C NMR ( $\delta$ /ppm): 171.09, 166.42, 152.98, 138.17, 131.36, 127.56, 123.28, 121.20, 112.00, 111.64, 111.03, 100.13, 55.34, 51.47, 39.83, 37.54; ESI-MS  $m/z$  = 339.2 ( $M+23$ )<sup>+</sup>; Anal. Calcd. for C<sub>17</sub>H<sub>20</sub>N<sub>2</sub>O<sub>4</sub>: C, 64.54; H, 6.37; N, 8.86, found: C, 64.70; H, 6.48; N, 8.77.

**Methyl 3-[(4-[(6-methoxyquinolin-8-yl)amino]pentyl]carbamoyl]but-3-enoate (11)** **11** was obtained by method B, from the reaction of monomethyl itaconate (0.072 g, 0.5 mmol), HATU (0.190 g, 0.5 mmol), DIEA (0.129 g, 1 mmol) and PQ base (0.142 g, 0.55 mmol) and after purification by column chromatography (dichloromethane/methanol = 95:5) and recrystallization from diethyl ether as a pale solid (0.110 g, yield 74%); mp 91–92 °C; IR (KBr) ( $\nu_{\max}$ /cm<sup>-1</sup>): 3404, 3340, 3002, 2962, 2928, 2862, 1763, 1658, 1622, 1578,

1522, 1450, 1388, 1342, 1226, 1202, 1170, 1054, 1008, 940, 824, 790, 748, 678, 628;  $^1\text{H}$  NMR ( $\delta$ /ppm): 8.53 (dd,  $J=4.2$ , 1.7 Hz, 1H), 8.15–8.02 (m, 2H), 7.42 (dd,  $J=8.3$ , 4.2 Hz, 1H), 6.47 (d,  $J=2.5$  Hz, 1H), 6.26 (d,  $J=2.5$  Hz, 1H), 6.12 (d,  $J=8.7$  Hz, 1H), 5.80 (s, 1H), 5.48 (s, 1H), 3.82 (s, 3H), 3.71–3.58 (m, 1H), 3.54 (s, 3H), 3.29 (s, 2H), 3.12 (dt,  $J=10.8$ , 6.6 Hz, 2H), 1.72–1.42 (m, 4H), 1.21 (d,  $J=6.2$  Hz, 3H);  $^{13}\text{C}$  NMR ( $\delta$ /ppm): 171.01, 166.42, 159.01, 144.63, 144.24, 138.13, 134.80, 134.53, 129.58, 122.10, 121.01, 96.12, 91.60, 54.98, 51.40, 46.99, 38.81, 37.53, 33.38, 25.90, 20.20; ESI–MS  $m/z=386.2$  ( $M+1$ ) $^+$ , 408.2 ( $M+23$ ) $^+$ ; Anal. Calcd. for  $\text{C}_{21}\text{H}_{27}\text{N}_3\text{O}_4$ : C, 65.44; H, 7.06; N, 10.90, found: C, 65.51; H, 7.00; N, 10.87.

**Methyl 3-([4-[(7-chloroquinolin-4-yl)amino]butyl]carbamoyl)but-3-enoate (12)** **12** was obtained by method B, from the reaction of monomethyl itaconate (0.072 g, 0.5 mmol), HATU (0.190 g, 0.5 mmol), DIEA (0.129 g, 1 mmol) and  $N^1$ -(7-chloroquinolin-4-yl)butane-1,4-diamine (0.137 g, 0.55 mmol) and after recrystallization from diethyl ether as a white solid (0.090 g, yield 48%); mp 94 °C (decomp.); IR (KBr) ( $\nu_{\text{max}}$ /cm $^{-1}$ ): 3418, 3278, 3242, 3120, 2956, 1742, 1706, 1610, 1552, 1452, 1388, 1362, 1318, 1214, 1166, 1102, 1016, 940, 842, 742;  $^1\text{H}$  NMR ( $\delta$ /ppm): 9.10 (t,  $J=5.6$  Hz, 1H), 8.52 (t,  $J=8.4$  Hz, 2H), 8.15 (t,  $J=5.7$  Hz, 1H), 7.89 (d,  $J=2.2$  Hz, 1H), 7.76 (dd,  $J=9.0$ , 2.2 Hz, 1H), 6.87 (d,  $J=7.0$  Hz, 1H), 5.80 (s, 1H), 5.50 (s, 1H), 3.54 (s, 7H), 3.18 (q,  $J=6.5$  Hz, 2H), 1.74–1.48 (m, 4H);  $^{13}\text{C}$  NMR ( $\delta$ /ppm): 171.04, 166.60, 154.75, 144.06, 139.72, 138.10, 137.49, 126.57, 125.32, 121.14, 120.14, 115.62, 98.67, 51.42, 42.85, 38.26, 37.51, 26.55, 24.92; ESI–MS  $m/z=376.1$  ( $M+1$ ) $^+$ ; Anal. Calcd. for  $\text{C}_{19}\text{H}_{22}\text{ClN}_3\text{O}_3$ : C, 60.72; H, 5.90; N, 11.18, found: C, 60.68; H, 5.99; N, 11.23.

**Methyl 3-([4-[[2,8-bis(trifluoromethyl)quinolin-4-yl]amino]butyl]carbamoyl)but-3-enoate (13)** **13** was obtained by method B, from the reaction of monomethyl itaconate (0.072 g, 0.5 mmol), HATU (0.190 g, 0.5 mmol), DIEA (0.129 g, 1 mmol) and  $N^1$ -[2,8-bis(trifluoromethyl)quinolin-4-yl]butane-1,4-diamine (0.193 g, 0.55 mmol) and after purification by column chromatography (dichloromethane/methanol=95:5) and recrystallization from diethyl ether as a pale solid (0.090 g, yield 48%); mp 115–116 °C; IR (KBr) ( $\nu_{\text{max}}$ /cm $^{-1}$ ): 3388, 3336, 2942, 1732, 1660, 1596, 1532, 1474, 1448, 1310, 1166, 1122, 1054, 1034, 952, 824, 772, 724, 668;  $^1\text{H}$  NMR ( $\delta$ /ppm): 8.65 (dd,  $J=8.7$ , 1.4 Hz, 1H), 8.14 (ddd,  $J=9.2$ , 6.5, 3.4 Hz, 2H), 7.99 (t,  $J=5.5$  Hz, 1H), 7.68 (t,  $J=7.9$  Hz, 1H), 6.88 (s, 1H), 5.80 (s, 1H), 5.49 (s, 1H), 3.54 (s, 3H), 3.41 (dd,  $J=7.2$ , 5.4 Hz, 2H), 3.30 (d,  $J=1.0$  Hz, 2H), 3.18 (q,  $J=6.5$  Hz, 2H), 1.76–1.63 (m, 2H), 1.63–1.54 (m, 2H);  $^{13}\text{C}$  NMR ( $\delta$ /ppm): 171.01, 166.53, 152.30, 148.36–147.38 (q,  $J=32.7$  Hz), 143.86, 138.13, 128.10–119.96 (q,  $J=272.0$  Hz), 128.92, 126.80–125.8 (q,  $J=28.8$  Hz), 124.43, 123.04, 121.01, 119.37, 94.19, 51.37,

42.27, 38.43, 37.49, 26.69, 24.95; ESI–MS  $m/z=478.1$  ( $M+1$ ) $^+$ ; Anal. Calcd. for  $\text{C}_{21}\text{H}_{21}\text{F}_6\text{N}_3\text{O}_3$ : C, 52.83; H, 4.43; N, 8.80, found: C, 52.88; H, 4.49; N, 8.84.

## General procedure for the synthesis of homodimers **14** and **15**

A solution of itaconic acid (0.065 g, 0.5 mmol), HATU (0.418 g, 1.1 mmol) and DIEA (0.184 g, 2.2 mmol) in dichloromethane (5 mL) was stirred at room temperature. After 10 min, a solution of the corresponding amine (1.15 mmol) in dichloromethane (5 mL) was added. The mixture was stirred at room temperature overnight and was then concentrated under reduced pressure. The residue was dissolved in ethyl acetate and washed with brine (3  $\times$  20 mL). The organic layer was dried with anhydrous sodium sulphate, filtered and concentrated under reduced pressure.

**$N'$ -[2-(5-methoxy-1H-indol-2-yl)ethyl]- $N$ -[2-(5-methoxy-1H-indol-3-yl)ethyl]-2-methylbut-2-enediamide (14)** **14** was obtained from the reaction of itaconic acid (0.065 g, 0.5 mmol), HATU (0.418 g, 1.1 mmol), DIEA (0.184 g, 2.2 mmol) and 2-(5-methoxy-1H-indol-3-yl)ethan-1-amine (0.219 g, 1.15 mmol) and after purification by column chromatography (cyclohexane/ethylacetate/methanol=1:1:0.5) and recrystallization from diethyl ether/petrolether as a white solid (0.038 g, yield 16%); mp 89–91 °C (decomp.); IR (KBr) ( $\nu_{\text{max}}$ /cm $^{-1}$ ): 3390, 3290, 3056, 2934, 1630, 1584, 1524, 1486, 1440, 1360, 1296, 1216, 1172, 1100, 1072, 1030, 922, 832, 798, 708, 638;  $^1\text{H}$  NMR ( $\delta$ /ppm): 10.63 (d,  $J=7.8$  Hz, 2H), 8.33 (t,  $J=5.7$  Hz, 1H), 8.13 (t,  $J=5.7$  Hz, 1H), 8.11, 7.23 (s, 1H), 7.20 (s, 1H), 7.13 (d,  $J=2.4$  Hz, 1H), 7.11 (d,  $J=2.4$  Hz, 1H), 7.04 ((d,  $J=2.4$  Hz, 1H), 7.02 (d,  $J=2.5$  Hz, 1H), 6.71 (d,  $J=2.2$  Hz, 1H), 6.69 (d,  $J=2.2$  Hz, 1H), 5.83 (s, 1H), 3.74 (s, 3H), 3.72 (s, 3H), 3.39–3.34 (m, 4H), 2.86–2.79 (m, 4H), 1.88 (s, 3H);  $^{13}\text{C}$  NMR ( $\delta$ /ppm): 168.94, 164.03, 152.97, 152.94, 144.74, 131.37, 127.54, 127.49, 123.37, 123.34, 121.82, 112.01, 111.97, 111.75, 111.56, 111.10, 110.99, 100.14, 100.05, 55.39, 55.29, 39.45, 25.19, 24.91, 21.98; ESI–MS  $m/z=497.4$  ( $M+23$ ) $^+$ ; Anal. Calcd. for  $\text{C}_{27}\text{H}_{30}\text{N}_4\text{O}_4$ : C, 68.34; H, 6.37; N, 11.81, found: 68.50; H, 6.21; N, 11.71.

**$N,N'$ -bis(4-[[2,8-bis(trifluoromethyl)quinolin-4-yl]amino]butyl)-2-methylbut-2-enediamide (15)** **15** was obtained from the reaction of itaconic acid (0.065 g, 0.5 mmol), HATU (0.418 g, 1.1 mmol), DIEA (0.184 g, 2.2 mmol) and  $N^1$ -[2,8-bis(trifluoromethyl)quinolin-4-yl]butane-1,4-diamine (0.403 g, 1.15 mmol) and after purification by column chromatography (dichloromethane/methanol=95:5) and recrystallization from diethyl ether/petrolether as a white solid (0.179 g, yield 45%); mp 92–94 °C (decomp.); IR (KBr) ( $\nu_{\text{max}}$ /cm $^{-1}$ ): 3452, 3340, 2928, 2872, 1664, 1634, 1596, 1552, 1444, 1362, 1314, 1224, 1140,

1044, 952, 822, 766, 722;  $^1\text{H}$  NMR ( $\delta/\text{ppm}$ ): 8.66–8.62 (m, 2H), 8.25 (t,  $J=5.7$  Hz, 1H), 8.14–8.09 (m, 2H), 8.04 (t,  $J=5.7$  Hz, 1H), 7.95 (dt,  $J=7.8$ , 5.3 Hz, 2H), 7.65 (m, 2H), 6.84 (d,  $J=5.1$  Hz, 2H), 5.80 (s, 1H), 3.41–3.33 (m, 4H), 3.13 (dq,  $J=16.9$ , 6.5 Hz, 4H), 1.88 (s, 3H), 1.76–1.47 (m, 8H);  $^{13}\text{C}$  NMR ( $\delta/\text{ppm}$ ): 168.94, 164.05, 152.30, 152.26, 148.37–147.39 (q,  $J=38.1$  Hz), 144.76, 143.84, 128.89, 128.07–119.93 (q,  $J=273.2$  Hz), 126.96, 126.96–125.93 (q,  $J=28.9$  Hz), 125.76–117.54 ( $J=275.6$  Hz), 124.41, 121.60, 119.34, 94.14, 42.36, 42.22, 38.12, 38.08, 26.65, 26.41, 24.99, 24.94, 21.93; ESI-MS  $m/z=797.4$  ( $\text{M}+1$ ) $^+$ ; Anal. Calcd. for  $\text{C}_{35}\text{H}_{32}\text{F}_{12}\text{N}_6\text{O}_2$ : C, 52.77; H, 4.05; N, 10.55, found: C, 52.89; H, 4.20; N, 10.66.

### Anticancer activity

Cancer cells of human solid tumours (MCF-7, HCT 116, H460, LN-229, Capan-1) were cultured as monolayers and maintained in the media recommended by the suppliers in a humidified atmosphere with 5%  $\text{CO}_2$  at 37 °C. The growth inhibition activity of the compounds was assessed as described in previous publication [17]. The HCT 116, H460 and MCF-7 cell lines were inoculated onto a series of standard 96-well microtiter plates on day 0, at  $3 \times 10^4$  cells/mL (HCT 116, H460) to  $5 \times 10^4$  cells/mL (MCF-7), depending on the growth of the tumour cell line. Test agents were then added in ten-fold dilutions ( $10^{-8}$  to  $10^{-4}$  M) and the cell cultures were then incubated for 72 h with 5%  $\text{CO}_2$  at 37 °C. The cell growth rate was evaluated by performing the MTT assay. The absorbance was measured on a microplate reader at 570 nm. MTS cell proliferation assays were performed for all other cell lines. Adherent cell lines LN-229 and Capan-1 cells were seeded at a density of 375 and 250 cells per well, respectively, in standard 384-well tissue culture plates. After overnight incubation, cells were treated with the test compounds at seven different concentrations ranging from 100 to  $6.4 \times 10^{-3}$   $\mu\text{M}$ . Suspension cell lines HL-60, K-562, Z-138, and DND-41 were seeded at densities ranging from 2500 to 6000 cells per well in 384-well tissue culture plates containing the test compounds at the same seven concentration points. Cells were incubated for 72 h and were then treated with the CellTiter 96<sup>®</sup> AQueous Non-Radioactive Cell Proliferation Assay reagent (Promega) according to the manufacturer's instructions. Absorbance of the samples was measured at 490 nm using a SpectraMax Plus 384 microplate reader (Molecular Devices).

For both assays, the absorbance is directly proportional to the number of living, metabolically active cells. The results are expressed as  $\text{GI}_{50}$ , which is the concentration necessary for 50% of inhibition. The  $\text{GI}_{50}$  values for each compound are calculated from concentration–response curves using linear regression analysis by fitting the test concentrations that give a percentage of growth (PG) values above and below

the reference value (i.e. 50%). If however, for all of the tested concentrations produced PGs were exceeding the respective reference level of effect (e.g. PG value of 50), then the highest tested concentration is assigned as the default value, which is preceded by a “>” sign. Two individual experiments were carried out and each test point was performed at least in triplicate.

### Antiviral activity

Antiviral activity of compounds **2–15** was determined as described previously [40]. Cytotoxicity and antiviral activity assay towards herpes simplex virus-1, herpes simplex virus-2, vaccinia virus, adenovirus-2, human coronavirus (229E) in HEL cell cultures, vesicular stomatitis virus, Coxsackievirus B4, respiratory syncytial virus in Hep-2 cell cultures, reovirus-1, sindbis virus, Punta Toro virus, yellow fever virus, Zika virus in Vero cell cultures and influenza A/H1N1 (A/Ned/378/05), influenza A/H3N2 (A/HK/7/87), influenza B (B/Ned/537/05) in MDCK cell cultures were performed. On the day of the infection, growth medium was aspirated and replaced by serial dilutions of the test compounds. The virus was then added to each well, diluted to obtain a viral input of 100  $\text{CCID}_{50}$  ( $\text{CCID}_{50}$  being the virus dose that is able to infect 50% of the cell cultures). Mock-treated cell cultures receiving solely the test compounds were included, to determine their cellular cytotoxicity. After 3 to 10 days of incubation, the virus-induced cytopathicity was determined by visual scoring of the cytopathic effect (CPE) (light microscopic evaluation of the virus-induced CPE and inhibition of evaluated compounds), as well as by measuring cell viability with the colorimetric formazan-based MTS assay. All experiments were performed in duplicate. Antiviral activity was expressed as  $\text{EC}_{50}$ . The activities were compared with the activities of the parent drugs PQ, CQ and MQ, DS-10.000 (dextran sulphate, approx. MW = 10.000) and standard antiviral drugs: ribavirin, mycophenolic acid, brivudin, cidofovir, aciclovir, ganciclovir, zalcitabine, alovudine, *Urtica dioica* agglutinin (UDA), zanamivir and rimantadine.

### Interaction with glutathion

A solution of compound **2** and **12**, respectively ( $c = 1$   $\mu\text{M}$ ) was incubated with glutathion solution ( $c = 125$   $\mu\text{M}$ ) in ammonium formate buffer ( $\text{pH} = 7.4$ )/acetonitrile (10%) at 37 °C for 6 days [26]. The progress of the reactions was monitored by MS. Aliquots of the reaction mixture (taken after 0, 5, 24, 48, 72, 96 and 144 h) were analyzed with Synapt G2-Si ESI-QTOF-MS system (Waters, Milford, USA). The aliquots were diluted 10 times with acetonitrile and sprayed at a flow rate of 50  $\mu\text{L}/\text{min}$  using the fluidics system of the instrument. MS conditions were set as follows: positive ion mode, capillary voltage 3 kV, sampling cone

voltage 10 V, source temperature 120 °C, desolvation temperature 350 °C, desolvation gas flow 800 L/h. Mass spectra were recorded from 100–1000 *m/z* at a frequency of 1 Hz. Data were acquired and analyzed with Waters MassLynx v4.1 software. The MS analysis confirmed the minimal consumption of the tested compounds with GSH, less than 4%.

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