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Chromophore-modified bis-benzo[*g*]indole carboxamides: synthesis and antiproliferative activity of bis-benzo[*g*]indazole-3-carboxamides and related dimers

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Dedicated to the memory of Professor Piero Pratesi

Abstract

Tricyclic pyrazole dimers that comprise two kinds of CONH–$(CH_2)_n$–$N(CH_3)$–$(CH_2)_n$–NHCO bridges to which are linked potential DNA-intercalating groups such as 1*H*-benzo[*g*]indazole, 2*H*-benzo[*g*]indazole and 1,4-dihydroindeno[1,2-*c*]pyrazole were designed, synthesized and some of them evaluated in vitro by NCI (Bethesda, USA) against nine types of cancer cells. Compounds **2a**, **2f**–**i** and **2o**–**r** demonstrated significant antiproliferative activity, all with GI_{50} values in the low micromolar range. Preliminary analysis of the structure–activity relationship for dimers **2** indicated that: (i) in the ground terms (**2a** and **2k**) antitumor activities were strongly related to the type of chromophore, (ii) in contrast, either 1*H*-benzo[*g*]indazole- or 1,4-dihydroindeno[1,2-*c*]pyrazole-dimers when bore a N_1-aryl group (**2g**, **2h**, **2i**, **2o**, **2p**, **2q** and **2r**) generally showed a good level of antitumor potency and (iii) for the most representative compounds (pairs of compounds: **2g**,**2h**; **2o**,**2p** and **2q**,**2r**) the length of the bridges did not significantly contribute to the variations in cytotoxicity. Two members of this series, **2f** and **2q**, were selected and tested in the hollow fiber cell assay to evaluate in a preliminary fashion their in vivo antitumor activity. Finally, viscosity measurement of **2f** with poly(dA-dT)$_2$, confirmed that these promising compounds behaved as typical DNA-intercalating agents.
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Keywords: Tricyclic pyrazole; Cytotoxic activity; Viscosity

1. Introduction

Cancer remains a major public health issue at the beginning of the 21st century. Because of this critical situation, much effort has been directed to the design of new drugs for cancer therapeutics. DNA-interactive drugs in current clinical use represent one of the most important drug classes in cancer therapy [1]. In general there are three major types of the above mentioned clinically important drugs: the intercalators, which insert between the base pairs of the double helix and determine a significant change of DNA conformation being accompanied by unwinding and elongation of the duplex; the alkylators, which react covalently with DNA bases; and the DNA strand breakers, which generate reactive radicals that produce cleavage of the polinucleotide strands [2].

Among intercalative substances it is also known [3] that molecular duplication transforms mono-intercalators into bifunctional intercalating agents (also called bis-intercalators) in which there are two potential intercalating groups (chromophores) tethered together. In general the affinity of these compounds for DNA is greater than that of their monointercalating counterparts, thus improving their antiproliferative activity.

As a result of this considerations, we have initiated a program focused on exploring bis-intercalators obtained by linking two benzo[*g*]indole groups with amino bis-alkylamide bridges and have reported that some deri-

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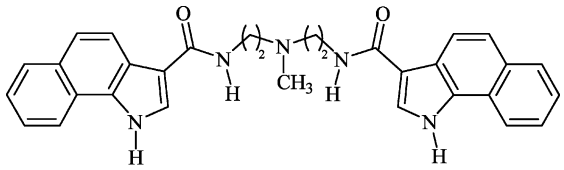


1, GI_{50} = 1.86 μM

Chart 1.

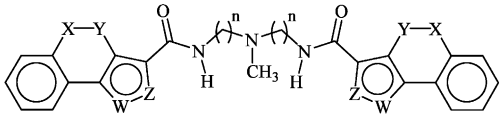
vatives, as represented by structure **1** (Chart 1), have good cytotoxicity against cancer cells in vitro tests [4].

In order to improve the activity and cell line selectivity, we planned to investigate, following the classical –CH=/–N= bioisosterism, aza-analogues **2** of the bis-benzo[*g*]indoles **1**, in particular exploring the effect produced by the presence of two 1*H*-benzo[*g*]indazole, 2*H*-benzo[*g*]indazole and 1,4-dihydroindeno[1,2-*c*]pirazole moieties with two different amino bis-alkylamide chains on cellular cytotoxicity. The influence on activity of groups at tricyclic nitrogens has also been explored (**2a**–**r**, Chart 2).

2. Chemistry

The preparation of tricyclic pyrazole bis-intercalators **2a**–**r** was carried out as shown in Scheme 1.

Acids **3**–**11** were activated with 1,1′-carbonyldiimidazole (CDI), and then reacted with a stoichiometric amount of the appropriate diamine in DMF at r.t. The carboxylic acid **3**–**11** were not known, with the exception of **10** [5], and were for the most part prepared using a similar method (Scheme 2).



2

2	X–Y	W–Z	n
a	CH=CH	HN–N	2
b	CH=CH	HN–N	3
c	CH=CH	CH_3N–N	2
d	CH=CH	CH_3N–N	3
e	CH=CH	N–NCH_3	2
f	CH=CH	N–NCH_3	3
g	CH=CH	C_6H_5N–N	2
h	CH=CH	C_6H_5N–N	3
i	CH=CH	*o,p*-Cl_2-C_6H_3N–N	2
j	CH=CH	*o,p*-Cl_2-C_6H_3N–N	3
k	CH_2	HN–N	2
l	CH_2	HN–N	3
m	CH_2	CH_3N–N	2
n	CH_2	CH_3N–N	3
o	CH_2	C_6H_5N–N	2
p	CH_2	C_6H_5N–N	3
q	CH_2	*o,p*-Cl_2-C_6H_3N–N	2
r	CH_2	*o,p*-Cl_2-C_6H_3N–N	3

Chart 2.

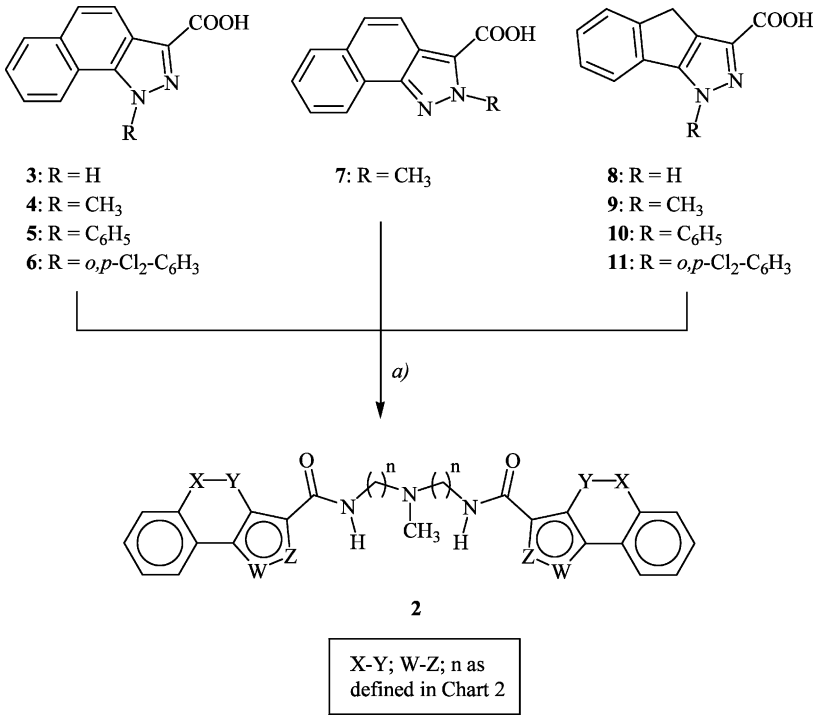
Regioselective cyclization of **14** and **15** with hydrazine hydrate, phenylhydrazine hydrochloride or 2,4-dichlorophenylhydrazine hydrochloride led to tricyclic pyrazoles **16**, **18**, **19**, **26**, **28** and **29** in good yields. In the case of methylhydrazine no regiospecificity was observed, and the cyclization steps resulted in the formation of two pairs of isomers, **17**/**20** and **27**/**30**, separable by silica gel flash-chromatography. These new compounds were fully characterized by spectroscopic techniques, namely ^1H, ^{13}C NMR and nOe difference experiments (Table 1). In the case of condensation of **14** the analysis of the NMR spectra allowed us to identify the compound with the lower R_f (55%) as the isomer **17** and the other as **20** (35%). The identification of compounds **17** and **20** was based mainly on their ^{13}C NMR spectra. Compound **17** shows the following features for the quaternary carbons in the heteroaromatic region: three singlets at 137.77, 137.91 and 139.56 ppm corresponding, respectively, to the pyrazole C-3a, C-3 and C-9b carbons. The ^{13}C NMR spectrum of compound **20** shows the aforementioned singlets at 135.98, 128.69 and 146.68 revealing a high-field shift for the C-3 carbon and a low-field shift for C-9b carbon in accord with simple mesomeric considerations and with published data [6] for similar systems.

In addition, the tricyclic pyrazoles showed similar ^1H NMR features in the aliphatic region, but differ significantly in the aromatic one in which the aromatic C_9–H doublet of **17** appeared at ca. 7.57 ppm. A remarkable feature in the ^1H NMR spectrum of **20** was the downfield shift of C_9–H, which adsorbed at ca. 7.82 ppm.

Finally, the identity of regioisomers **17** and **20** was unequivocally proved by nOe difference spectroscopy analysis. Irradiation of the resonance frequency of the protons C_9-H led to the enhancement of the signal of the methyl protons CH_3–N_1 in the case of regioisomer **17**, whereas for regioisomer **20** no enhancement of the same signal was observed.

When cyclocondensation was carried out with **15** and methylhydrazine a mixture of regioisomers was formed. After work up and on the basis of simple analysis on silica gel thin layer chromatography (TLC) of the crude product, we empirically correlated the structure of the slow-moving **27** with **17** and that of **30** with **20**. We were able to establish by ^1H and ^{13}C NMR spectra the structure **27** for the main product (55%, lower R_f) and the structure **30** for the minor one (10%). These assignments were confirmed by the nOe experiments with isomers **27** and **30**. Thus, C_8–H proton of the aromatic moiety of **27** located in the close proximity of N_1–CH_3 exhibited nOe enhancement whereas no interaction was seen between these two groups for **30**.

The treatment of compounds **16**–**20** with 2,3-dichloro-5,6-dicyano-*p*-benzoquinone (DDQ) gave the



2

Scheme 1. Reagents and conditions: (a) CDI, DMF, 3 h, r.t. then $CH_3N(CH_2CH_2NH_2)_2$ or $CH_3N(CH_2CH_2CH_2NH_2)_2$, DMF, 48 h, r.t.

esters **21**–**25**. The esters **21**–**29** were hydrolized in basic solution to afford acids **3**–**11**.

The α,γ-diketoesters **14** [5] and **15**, isolated as hydroxymethylene tautomers, were prepared in excellent yield (>90%) from the requisite benzocyclanones **12** and **13** and ethyl oxalate in the presence of sodium ethoxide.

3. Results and discussion

Compounds **2a**,**f**–**i**,**k**–**r** were selected by the US National Cancer Institute (NCI) for evaluation in an in vitro preclinical antitumor screening program [7–9] against ca. 60 human tumor cell lines derived from leukemia, non-small cell lung cancer, colon cancer, central nervous system (CNS) cancer, melanoma, ovarian cancer, renal cancer, prostate cancer and breast cancer. The results are summarized in Table 2 as cytostatic/cytotoxic parameters.

The cytostatic parameters include GI_{50} and TGI, which are the concentration of drug required for 50% growth inhibition and total growth inhibition, respectively. The cytotoxic parameter is the LC_{50}, which is the concentration required for 50% cell kill.

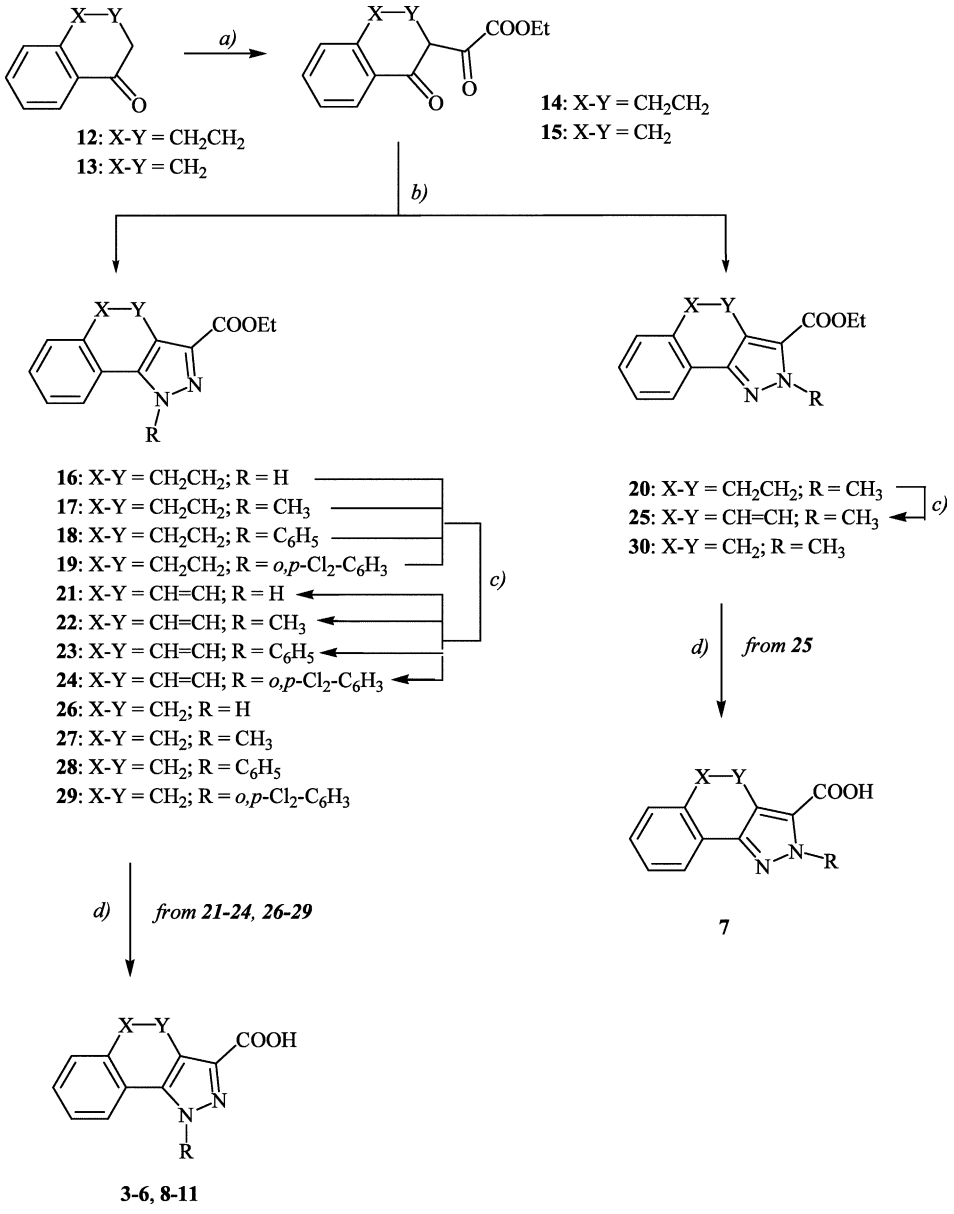
Table 3 lists GI_{50} values which, in those cell lines are lower than the mean values over all cell lines tested.

Initial substitution of the benzo[*g*]indole chromophore of **1** with 1*H*-benzo[*g*]indazole (**1** versus **2a**) provided an active compound even if 1.38-fold less potent than **1**, with GI_{50} and TGI in the low micromolar range. Moreover, **2a** showed a wide-spectrum inhibitory effect on cancer cell growth with molar concentration ranging from 1.51 to 11.7 μM in almost all cell lines.

The GI_{50} values obtained for *N*-aryl derivatives **2g**–**i** clearly showed that this substitution favourably affected the anticellular activity being 1.32–1.59 times more potent than the *N*-unsubstituted counterpart **2a**. Compounds **2g**–**i** exhibited potent antiproliferative activity against leukaemia cell lines with GI_{50} values up to 0.014 μM. Again, these compounds showed moderate-to-good activities against certain solid tumor cell lines. In addition the effect of the variation of benzo[*g*]indazole conjugation could be recognized by comparing the cytotoxicities of **2a** and **2f**. The 2*H*-benzo[*g*]indazole analogue **2f** did have respectable cytotoxicity (GI_{50} = 5.01 μM), but it was less active than the ground term **2a**. Compound **2f** showed potent selective cytotoxicity against all melanoma cell lines; however, **2f** was not cytotoxic against almost all other tumor cells.

Further structure–activity relationship studies focused on the effects of variation of aromatic tricyclic moiety of **2a** were then examined. *N*-unsubstituted 1,4-dihydroindeno[1,2-*c*]pyrazole analogue **2k** was found to be moderately cytostatic (GI_{50} = 18.20 μM) and only weakly cytocidal (LC_{50} = 69.18 μM).

The effect of the length of the bridge connecting the two chromophores could be recognized in this series by comparing the cytotoxicities of **2k** (GI_{50} = 18.20 μM) with **2l** (GI_{50} = 12.02 μM) and **2m** (GI_{50} = 15.49 μM) with **2n** (GI_{50} = 11.48 μM). This indicated a slight increase in biological activity as the length of the bridge was increased. Moreover, a direct comparison between

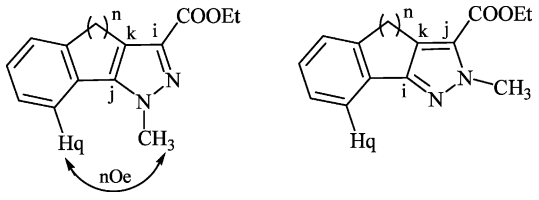


Scheme 2. Reagents and conditions: (a) EtONa, EtOOC–COOEt, 2–8 h, r.t. (b) EtOH, $RNHNH_2$, 2–5 h, reflux. (c) CH_2Cl_2, DDQ, 5 min, r.t. (d) MeOH, KOH, overnight, reflux.

2k and **2m** or **2l** and **2n** allowed a determination of the effect of replacing the NH of **2k** and **2l** with an N–CH_3 group, since the two pairs of structures were otherwise identical. The similar GI_{50} values for methyl derivatives **2l** and **2n** clearly showed that substitution at this position was tolerated. Turning to the N-aryl analogues, compounds **2o**–**r**, containing phenyl or 2,4-dichlorophenyl groups, showed good antiproliferative activities. Compound **2q** demonstrated improved cytotoxicity especially against leukaemia, colon cancer and melanoma cell lines, with GI_{50} values ranging from 1.17 to 2.31 μM. Moreover, TGI values supported that compound **2q** had high inhibitory activity against both colon cancer cells (TGI = 4.68 μM) and melanoma cancer cells (TGI = 4.01 μM) (panel selectivity).

Based on in vitro anti-cancer screening results, NCI selected compounds **2f** and **2q** for preliminary in vivo hollow-fiber assay [10]. Each compound was tested against a standard panel of 12 human tumor cell lines, including NCI-H23, NCI-H522, MDA-MB-231, MDA-MB-435, SW-620, COLO 205, LOX IMVI, UACC-62, OVCAR-3, OVCAR-5, U 251 and SF-295. According to the NCI's protocol, compounds with a combined intraperitoneal (IP) and subcutaneous (SC) score of 20, a SC score 8 or a net cell kill of one or more cell lines in either implant site, are referred for xenograft testing.

Table 1
Determination of the regiochemistry of isomers **17/20** and **27/30**



Comp.	*n*	R_f [a]	Yield (%)	$\delta_{C\text{-}k}$	$\delta_{C\text{-}i}$	$\delta_{C\text{-}j}$	$\delta_{H\text{-}q}$
17	2	0.27	55	137.77	137.91	139.56	7.57
27	1	0.23	55	148.84	150.10	136.73	7.53
20	2	0.76	35	135.98	146.68	128.69	7.82
30	1	0.69	10	147.50	157.68	129.86	7.75

^{13}C and ^{1}H NMR spectra were recorded in $CDCl_3$.
[a] R_f values were determined on TLC using Polygram® SIL N-HR-/HV_{254} precoated plastic sheet (0.2 mm) with 4:1 petroleum ether/ethyl acetate as eluent.

The results are: **2f**, IP = 2, SC = 0, cell kill = 0; **2q**, IP = 10, SC = 6, cell kill = 0. Only 1,4-dihydroindeno[1,2-*c*]pyrazole derivative **2q** produced a moderate reduction in the viable cell mass below the level present at the start of implantation.

In order to determine at the molecular level whether compounds **2** have the ability to intercalate into DNA strands, the relative viscosity (η/η_0) of polynucleotide Poly(dA-dT)·poly(dA-dT) in the presence of the ground term **2f** (used as $3HCl\cdot 3H_2O$ derived), Ethidium bromide (EtBr), and Distamycin A (Dist) were measured. It is known that EtBr increased the viscosity of DNA, by intercalation in DNA base pairs, while Dist, a groove binding to DNA, did not affect the viscosity significantly [11].

The data of Fig. 1 show that the binding of **2f** with Poly(dA-dT)·poly(dA-dT) caused a curve profile similar to that of EtBr, thus demonstrating, for this class of compounds, an intercalative mode of DNA interaction.

In summary, evaluation of a number of tricyclic pyrazole dimers for cytotoxicity revealed that the greatest activity resided in compounds incorporating *N*-aryl substituents. In particular, **2q** is a prototypic molecule serving as a template for subsequent molecular modification with a view to increased activity and selective toxicity for colon and melanoma neoplasms.

Table 2
Inhibition of in vitro tumor cell growth[a] by **2a**, **f**–**i**, **k**–**r** and **1**

Comp.	Log GI_{50}	GI_{50} (μM) [b]	Log TGI	TGI (μM) [b]	Log LC_{50}	LC_{50} (μM) [c]
2 [a]	−5.59	2.57	−5.19	6.45	−4.46	34.6
2f [d]	−5.30	5.01	−4.86	13.80	−4.44	36.3
2g [d]	−5.71	1.95	−5.29	5.13	−4.83	14.8
2h [d]	−5.79	1.62	−5.41	3.89	−4.21	61.7
2i [d]	−5.79	1.62	−5.49	3.24	−5.11	7.76
2k	−4.74	18.20	−4.44	36.31	−4.16	69.18
2l	−4.92	12.02	−4.59	25.70	−4.26	54.95
2m	−4.81	15.49	−4.47	33.88	−4.17	67.61
2n [e]	−4.94	11.48	−4.52	30.20	−4.15	70.79
2o	−5.73	1.86	−5.40	3.98	−5.08	8.32
2p	−5.85	1.41	−5.52	3.02	−5.18	6.61
2q	−5.54	2.88	−4.89	12.88	−4.32	47.86
2r	−5.81	1.55	−5.50	3.16	−5.21	6.17
1	−5.73	1.86	−5.35	4.47	−4.97	10.71

[a] Expressed as meangraph midpoint (MG-MID).
[b] GI_{50} and TGI are the concentration in μmoles per liter required for 50 and 100% growth inhibition, respectively (cytostatic effect).
[c] LC_{50} is the concentration in μmoles per liter required for 50% lethality (cytocidal effect). The three parameters are reported both in logarithmic and arithmetical scales.
[d] As hydrochloride salt.
[e] As fumarate salt.

Table 3
Inhibition of in vitro tumor cell growth by **2a**, **f**–**i**, **k**–**r** and **1**

Panel/cell line	Antiproliferative activity (GI_{50} in μM) [a]													
	2a	**2f**	**2g**	**2h**	**2i**	**2k**	**2l**	**2m**	**2n**	**2o**	**2p**	**2q**	**2r**	**1**
Leukemia														
CCRF-CEM	2.29	–	–	0.014	–	18.1	11.1		2.18	1.47	0.87	1.74	0.60	1.48
HL-60 (TB)	–	–	–	0.035	0.32	–	1.67	12.9	2.04	1.24	0.31	1.17	1.41	
K-562		2.59		–	1.10	18.0		12.7	6.01	–	0.36	2.00	0.54	1.82
MOLT-4		3.33	0.38	–	1.61	–	10.4	12.4	5.36	–	1.08	2.12	1.32	1.73
RPMI-8226	2.12	1.78	0.20	–	–					–		1.62	1.20	
SR					0.28	–			2.99		0.19			1.84
Non-small cell lung cancer														
A549/ATCC	2.20	–	–	–	–	–	–	–	–	–	–	–	–	1.46
EKVX	1.65	–	–	–	–	–	–	–	–	1.75	–	–	–	1.81
HOP-62	2.38	–	1.43	–	–	14.8	–	15.4	9.09	1.84	–	–	–	–
HOP-92	–	4.26	1.73	–	–	17.3	–	12.9	–	1.68	–	1.81	–	1.54
NCI-H226	1.92	–	–	–	–	–	–	–						
NCI-H23	1.90	–	–	–	–	17.2	–	15.4	–	–	–	–	–	1.54
NCI-H322M	2.11	–	–	–	–		–	–	–	1.65	–	–	–	1.75
NCI-H460	2.56	3.70	1.41	–	–	18.1	–	–	–	1.73	–	1.77	–	–
NCI-H522	1.98	4.41	–	–	1.46	17.5	–	–		1.71	1.30	1.51	–	1.71
Colon cancer														
COLO 205	1.51	1.63	1.54	1.44	–	17.3	–	11.9	–	1.74	1.27	1.90	–	1.44
HCC-2998	1.68	1.60	1.53	1.31	1.53	15.4	–	12.5	9.80	1.71	1.41	1.74	1.52	1.41
HCT-116	2.27	4.23	1.53	–	–	16.2	–	15.1	–	1.63	–	2.13	–	1.61
HCT-15	–	4.14	1.76	–	–	–	–	–	–	–	–	2.15	–	1.64
HT29	2.24	1.64	–	–	1.57	17.6	–	14.0	–	1.76	–	2.02	–	1.76
KM12	1.78	–	–	–	–	17.3	–	–	–	1.83	–		–	1.63
SW-620	1.88	4.76	–	–	–	–	–	–		1.25		1.50	1.18	1.71
CNS cancer														
SF-268	2.17	–	–	–	1.61	–	–	14.7	–	1.76	–	–	–	–
SF-295	1.84	1.75	1.61	–	–	16.6	–	–	–	1.85	–	2.75	1.17	–
SF-539	1.79	2.81	1.66	–	–		11.0	14.3	–	–	–	1.75	1.19	0.57
SNB-19	1.83	–	–	–	1.54	16.8	–	–	7.22	–	–	–	–	1.48
SNB-75	1.84	–	–	–	–	12.5	–	14.4	–	1.48	–	–	1.32	1.25
U251	2.05	4.98	1.57	–	–	–	–	–	11.1	1.81	–	–	–	1.76
Melanoma														
LOX IMVI	1.81	1.87	1.78	–	–	–	–	–	2.55	1.86	1.37	1.85	–	1.48
MALME-3M	1.73	2.93	–	–	–	–	10.3	–		1.83	–	1.12	–	–
M14	1.93	2.15	1.78	–	1.60	–	–	12.6	2.27	1.85	0.67	1.76	–	1.46
SK-MEL-2	–	2.83	1.86	–	–		10.6	14.7	–	–	1.40	2.31	–	1.60
SK-MEL-28	2.00	1.85	1.70	–	–	–	–	–	–	1.70	–	1.74	1.53	1.67
SK-MEL-5	1.75	1.67	1.64	–	–	16.9	–	12.1	–	1.83	–	1.69	–	–
UACC-257	–	1.90	1.71	–	–		–	14.0	–	1.63	–	1.84	–	1.82
UACC-62	1.98	1.72	1.77	1.55	–		–	15.2	–	1.75	–	1.84	–	1.73
Ovarian cancer														
IGROV1	1.68	–	–	–	–	16.3	–	8.56	–	1.67	1.39	–	–	1.47
OVCAR-3	1.91	–	1.70	–	–	14.9	–	11.4	–	1.78	–	–	–	–
OVCAR-4	2.04	–	–	–	–	15.9	–	–	–	1.74	–	0.75	0.053	–
OVCAR-5	1.87	–	–	–	–	–	–	–	–	1.79	–	–	–	–
OVCAR-8	2.12	–	1.79	–	–	–	–	–	–	1.78	–	1.80	–	1.74
SK-OV-3	2.01	–	–	–	–	16.5	–	–	–	–	–	–	–	–
Renal cancer														
786-0	1.75	–	1.67	–	1.54	16.0	10.9	14.5	9.13	1.70	–	2.27	–	1.54
A498	2.11	3.06	–	–	1.43	–	–	15.3	–	1.86	–	2.53	–	1.53
ACHN	1.91	–	–	–	–	17.1	–	14.5	–	1.72	–	–	–	1.62
CAKI-1	–	3.65	–	–	1.58	16.7	–	–	–	–	–	1.66	–	–
RXF 393	2.02	4.89	1.62	–	–	15.4	–	14.8	–	1.43	–	2.67	–	1.14
SN12C	1.83	–	1.88	–	–	16.7	–	14.9	–	1.86	–	–	–	–
TK-10			1.40	–	–	–	–	–	–	1.72	–	–	–	–
UO-31	–	4.62	–	1.46		–	–	10.4	0.27	1.54	–	–	–	1.38

Table 3 (*Continued*)

Panel/cell line	Antiproliferative activity (GI_{50} in μM) [a]													
	2a	**2f**	**2g**	**2h**	**2i**	**2k**	**2l**	**2m**	**2n**	**2o**	**2p**	**2q**	**2r**	**1**
Prostate cancer														
PC-3	–	–	–	1.60	–	17.8	–	–	–	–	–	–	–	1.59
DU-145	2.16	–	–	–	–	15.5	–	–	–	–	–	–	–	1.32
Breast cancer														
MCF7	2.07	3.13	–	–	1.56	–	–	11.1	–	1.67	–	2.66	1.55	1.43
NCI/ADR-RES	–	–	–	–	1.59	–	10.0	–	–	–	–	–	–	1.79
MDA-MB-231/ATCC			1.29	–	–	–	10.9	–	–	–	–	–	–	1.35
HS 578T			–	–	1.44	–	–			–		1.53	–	–
MDA-MB-435	1.94	1.95	–	–	–	17.5	–	15.3	10.6	1.57	1.22	0.86	1.25	–
MDA-N	1.86	2.21	–	–	–	16.5	–	14.5	–	1.74	1.36	2.33	–	1.85
BT-549	–	–	1.02	–	–	16.8	–	–	–	–	–	–	–	–
T-47D	2.20	4.47	–	–	–	–	–	–	–	–	–	–	–	1.17

[a] Data obtained from NCI in vitro disease oriented tumor cell screen.

4. Experimental

Melting points were obtained on an Electrothermal IA 9100 digital melting point apparatus or on a Köfler melting point apparatus and are uncorrected. IR spectra were recorded as thin films (for oils) or nujol mulls (for solids) on NaCl plates with a Perkin Elmer 781 IR spectrophotometer and are expressed in ν (cm^{-1}). UV–Vis spectra were recorded as ethanolic solution with a Perkin Elmer Lambda 5 spectrophotometer and the absorption wavelengths are expressed as λ_{max} in nm followed by log ε. All NMR spectra were taken on a Varian XL-200 NMR spectrometer with ^{1}H and ^{13}C being observed at 200 and 50 MHz respectively. Chemical shifts for ^{1}H and spectra were reported in δ or ppm downfield from TMS [$(CH_3)_4Si$]. Multiplicities are recorded as s (singlet), br s (broad singlet), d (doublet), t (triplet), q (quartet), qu (quintuplet), m (multiplet). Elemental analyses were performed by Laboratorio di Microanalisi, Dipartimento di Chimica, Università di Sassari, Italy and are within ±0.4% of the calculated values. All reactions involving air or moisture-sensitive compounds were performed under argon atmosphere.

The general procedure for conversion to an HCl salt was the addition of excess ethereal HCl solution to a solution of the compound in chloroform or diethyl ether. The solvent was evaporated and the resulting salt was triturated with anhydrous ether and dried on vacuum.

The general procedure for conversion to a fumarate salt was the addition of a stoichiometric amount of a solution of fumaric acid in dry methanol to a solution of the compound in dry methanol. The solvent was evaporated and the resulting salt was triturated with anhydrous ether and dried on vacuum.

Unless otherwise specified, all materials, solvents, reagents and precursors **12**,**13** were obtained from commercial suppliers.

Flash chromatography (FC) was performed using Merck silica gel 60 (230–400 mesh ASTM). TLC was

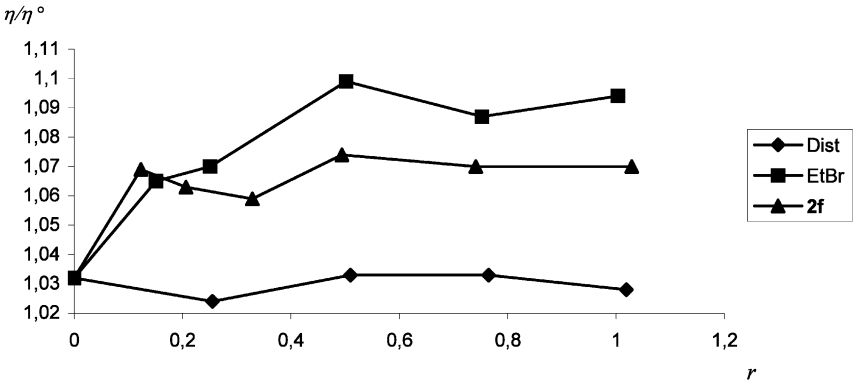


Fig. 1. Viscometric titration of Poly(dA-dT)·poly(dA-dT) with **2f**, EtBr and Dist.

performed with Polygram® SIL N-HR/HV$_{254}$ precoated plastic sheets (0.2 mm).

4.1. General procedure for preparation of ethyl 2-oxo-2-(1-oxo-1,2,3,4-tetrahydro-naphthalen-2-yl) acetate (**14**) and ethyl 2-oxo-2-(1-oxo-2,3-dihydro-1H-inden-2-yl) acetate (**15**)

Sodium metal (12 mmol) was added in small portion to dry ethanol (5 ml) and stirred until all the sodium had reacted. Ethyl oxalate (6 mmol) was added, follow by dropwise addition of a solution of appropriate ketone [**12**, **13**] starting material (6 mmol) in dry ethanol (30 ml). The solution was stirred at room temperature for 2–8 h. The mixture was slowly poured over 2 N hydrochloride acid and the resulting precipitate was collected by filtration and washed with a small volume of ice-cooled ethanol and water. The air-dried residue afforded the analytically pure product [**14**, **15**].

14: 98% yield; m.p. 43–45 °C (triturated with petroleum ether) ([12]: 44–45 °C).
15: 96% yield; m.p. 68–70 °C (triturated with petroleum ether) ([5]: 69–70 °C).

4.2. General procedure for preparation of dihydro-1H-benzo[g]indazole-carboxylate **16**–**20** and dihydro-indeno[1,2-c]pyrazole-carboxylates **26**–**30**

A stirred mixture of the appropriate diketoester (4 mmol) [**14**,**15**] and the requisite hydrazine hydrochloride (4.6 mmol) in EtOH (28 ml) was heated under reflux for 2–5 h. The reaction was allowed to cool to room temperature and the insoluble material was collected by filtration and washed with a small volume of ice-cooled ethanol. Purification by FC afforded the analytically pure product.

4.2.1. Ethyl 4,5-dihydro-1H-benzo[g]indazole-3-carboxylate (**16**)

Yield: 78%; yellowish solid; m.p. 144–145 °C (triturated with ethyl acetate) ([13]: 152 °C from ethanol).

4.2.2. Ethyl 1-methyl-4,5-dihydro-1H-benzo[g]indazole-3-carboxylate (**17**) and ethyl 2-methyl-4,5-dihydro-2H-benzo[g]indazole-3-carboxylate (**20**)

2,4-Diketoester **14** was transformed to a mixture of compounds **17** and **20** separated by FC eluting with petroleum ether/ethyl acetate 8:2.

17: Yield: 55%; white solid; m.p. 107–108 °C; R_f (petroleum ether/ethyl acetate 8:2) 0.27; IR (nujol): 1710 (C=O); UV (ethanol): λ_{max} 252 (3.88), 260 (3.87), 270 (3.74), 282 (3.69); ^{1}H NMR ($CDCl_3$): 1.43 (t, 3H, $J = 7$ Hz), 2.88–3.05 (m, 4H), 4.24 (s, 3H), 4.43 (q, 2H, $J = 7$ Hz), 7.25–7.40 (m, 2H), 7.55–7.62 (m, 2H). ^{13}C NMR ($CDCl_3$): 14.36 (CH_3), 19.80 (CH_2), 30.22 (CH_2), 40.21 (N_1–CH_3), 60.61 (CH_2), 122.02 (C), 122.08 (CH), 126.45 (C), 126.72 (C), 127.90 (CH), 128.95 (CH), 137.55 (C), 137.74 (C), 139.48 (C), 162.72 (C=O). *Anal*. Calc. for $C_{15}H_{16}N_2O_2$: C 70.29, H 6.29, N 10.93. Found: C 69.97, H 6.57, N 10.76%.

20: Yield: 35%; oil; b.p. 94 °C (0.1 mmHg); R_f (petroleum ether/ethyl acetate 8:2) 0.76; IR (film): 1715 (C=O); UV (ethanol): λ_{max} 226 (4.23), 243 (4.14), 282 (3.76); ^{1}H NMR ($CDCl_3$): 1.41 (t, 3H, $J = 7$ Hz), 2.88–3.10 (m, 4H), 4.21 (s, 3H), 4.38 (q, 2H, $J = 7$ Hz), 7.20–7.35 (m, 2H), 7.80–7.85 (m, 2H). ^{13}C NMR ($CDCl_3$): 14.13 (CH_3), 20.34 (CH_2), 28.82 (CH_2), 39.70 (N_2-CH_3), 60.61 (CH_2), 121.88 (CH), 122.06 (C), 126.69 (CH), 127.48 (CH), 128.09 (CH), 128.69 (C), 135.98 (C), 136.22 (C), 146.68 (C), 160.33 (C=O). *Anal*. Calc. for $C_{15}H_{16}N_2O_2$: C 70.29, H 6.29, N 10.93. Found: C 70.12, H 5.94, N 11.21%.

4.2.3. Ethyl 1-phenyl-4,5-dihydro-1H-benzo[g]indazole-3-carboxylate (**18**)

Yield: 78%; yellowish solid; m.p. 157–158 °C (ethanol) ([13]: 163 °C from ethanol).

4.2.4. Ethyl 1-(2,4-dichlorophenyl)-4,5-dihydro-1H-benzo[g]indazole-3-carboxylate (**19**)

Yield: 58%; yellowish solid; m.p. 131–132 °C (ethanol); R_f (petroleum ether/ethyl acetate 8:2) 0.59; IR (nujol): 1710 (C=O); UV (ethanol): λ_{max} 217 (4.44), 265 (4.05), 278 (4.02), 290 (3.91), 299 (3.98); ^{1}H NMR ($CDCl_3$): 1.43 (t, 3H, $J = 7$ Hz), 2.95–3.15 (m, 4H), 4.45 (q, 2H, $J = 7$ Hz), 6.57 (d, 1H, $J = 7.2$ Hz), 7.02 (t, 1H, $J = 7.2$ Hz), 7.19 (t, 1H, $J = 7.2$ Hz), 7.28 (t, 1H, $J = 7.2$ Hz), 7.40–7.60 (m, 3H). *Anal*. Calc. for $C_{20}H_{16}Cl_2N_2O_2$: C 62.03, H 4.16, Cl 18.31, N 7.23. Found: C 61.74, H 3.85, Cl 17.98, N 6.87%.

4.2.5. Ethyl 1,4-dihydro-indeno[1,2-c]pyrazole-3-carboxylate (**26**)

Yield: 31%; yellowish solid; m.p. 168 °C (from ethanol) ([13]: 174 °C from ethanol).

4.2.6. Ethyl 1-methyl-1,4-dihydro-indeno[1,2-c]pyrazole-3-carboxylate (**27**) and ethyl 2-methyl-1,4-dihydro-indeno[1,2-c]pyrazole-3-carboxylate (**30**)

2,4-Diketoester **15** was transformed to a mixture of compounds **27** and **30** separated by FC eluting with petroleum ether/ethyl acetate 8:2.

27: Yield: 55%; white solid; m.p. 178–179 °C (ethanol). R_f (petroleum ether/ethyl acetate 8:2) 0.23. IR (nujol): 1730 (C=O). UV (ethanol): λ_{max} 220 (4.13), 245 (4.12), 260 (4.05), 268 (3.93), 280 (3.91), 353 (3.33). ^{1}H NMR ($CDCl_3$): 1.43 (t, 3H, $J = 7.2$ Hz), 3.72 (s, 2H), 4.18 (s, 3H), 4.43 (q, 2H, $J = 7.2$ Hz), 7.25–7.40 (m, 2H), 7.52–7.56 (m, 2H). ^{13}C NMR ($CDCl_3$): 14.36 (CH_3), 29.58 (CH_2), 38.39 (N_1–CH_3), 60.75 (CH_2), 118.34 (CH), 126.35 (CH), 126.50 (CH), 126.75 (CH), 129.39

(C), 131.38 (C), 136.73 (C), 148.84 (C), 150.10 (C), 160.03 (C=O). *Anal*. Calc. for $C_{14}H_{14}N_2O_2$: C 69.41, H 5.82, N 11.56. Found: C 69.47, H 5.62, N 11.47%.

30: Yield: 10%; white solid; m.p. 98–100 °C. R_f (petroleum ether/ethyl acetate 8:2) 0.69. IR (nujol): 1710 (C=O). UV (ethanol): λ_{max} 243 (4.18), 275 (3.65), 295 (3.72). ^{1}H NMR ($CDCl_3$): 1.43 (t, 3H, $J = 7.2$ Hz), 3.77 (s, 2H), 4.27 (s, 3H), 4.39 (q, 2H, $J = 7.2$ Hz), 7.25–7.40 (m, 2H), 7.49–7.76 (m, 2H). ^{13}C NMR ($CDCl_3$): 14.30 (CH_3), 30.31 (CH_2), 39.75 (N_2–CH_3), 60.86 (CH_2), 119.82 (CH), 125.68 (CH), 126.69 (CH), 127.01 (CH), 129.86 (C), 134.40 (C), 147.50 (2 × C), 157.68 (C), 160.03 (C=O). *Anal*. Calc. for $C_{14}H_{14}N_2O_2$: C 69.41, H 5.82, N 11.56. Found: C 69.54, H 5.52, N 11.68%.

4.2.7. Ethyl 1-phenyl-1,4-dihydro-indeno[1,2-c]pyrazole-3-carboxylate (**28**)

Yield: 70%; yellowish solid; m.p. 111–112 °C (ethanol) ([14]: 110–112 °C from CH_2Cl_2/diisopropyl ether).

4.2.8. Ethyl 1-(2,4-dichlorophenyl)-1,4-dihydro-indeno[1,2-c]pyrazole-3-carboxylate (**29**)

Yield: 80%; yellowish solid; m.p. 165 °C (ethanol). ([15]: 165 °C triturated with ethyl acetate).

4.3. General procedure for preparation of benzo[g]indazole-carboxylates **21**–**25**

A stirred mixture of the appropriate ester (1.0 mmol) [**16**–**20**] and of DDQ (4.2 mmol) in CH_2Cl_2 (8 ml) was heated under reflux for 12 h. The reaction was allowed to cool to room temperature, taken up with CH_2Cl_2, washed with a 3% NH_4OH aqueous solution, dried (Na_2SO_4) and concentrated in vacuum. The residue was purified by FC affording the analytically pure product.

4.3.1. Ethyl 1H-benzo[g]indazole-3-carboxylate (**21**)

Yield: 82%; yellowish solid: M.p. 201–202 °C ([16]: 211 °C).

4.3.2. Ethyl 1-methyl-1H-benzo[g]indazole-3-carboxylate (**22**)

Yield: 91%; white–cream solid; m.p. 113–114 °C. R_f (petroleum ether/ethyl acetate 8:2) 0.24. IR (nujol): 1700 (C=O). UV (ethanol): λ_{max} 223 (4.01), 254 (3.53), 267 (3.46), 278 (3.49), 303 (3.09), 319 (3.14). ^{1}H NMR ($CDCl_3$): 1.51 (t, 3H, $J = 7.2$ Hz), 4.55 (q, 2H, $J = 7.2$ Hz), 4.63 (s, 3H), 7.60–7.75 (m, 2H), 7.99–8.05 (m, 2H), 8.20 (m, 1H), 8.40–8.50 (m, 1H). *Anal*. Calc. for $C_{15}H_{14}N_2O_2$: C 70.85, H 5.55, N 11.02. Found: C 70.79, H 5.86, N 11.38%.

4.3.3. Ethyl 1-phenyl-1H-benzo[g]indazole-3-carboxylate (**23**)

Yield: 86%; pink solid; m.p. 106–107 °C. R_f (CH_2Cl_2) 0.41. IR (nujol): 1710 (C=O). UV (ethanol): λ_{max} 219 (4.36), 236 (4.28), 250 (3.98), 290 (3.62), 321 (3.59), 355 (3.62). ^{1}H NMR ($CDCl_3$): 1.51 (t, 3H, $J = 7.0$ Hz), 4.57 (q, 2H, $J = 7.0$ Hz), 7.20–8.25 (m, 11H). *Anal*. Calc. for $C_{20}H_{16}N_2O_2$: C 75.93, H 5.10, N 8.86. Found: C 75.69, H 4.86, N 9.13%.

4.3.4. Ethyl 1-(2,4-dichlorophenyl)-1H-benzo[g]indazole-3-carboxylate (**24**)

Yield: 54%; pink solid; m.p. 136–137 °C. R_f (CH_2Cl_2) 0.54. IR (nujol): 1710 (C=O). UV (ethanol): λ_{max} 219 (2.92), 241 (2.78), 276 (2.37), 306 (1.98), 320 (1.98). ^{1}H NMR ($CDCl_3$): 1.52 (t, 3H, $J = 7.0$ Hz), 4.58 (q, 2H, $J = 7.0$ Hz), 7.25–8.35 (m, 9H). *Anal*. Calc. for $C_{20}H_{14}Cl_2N_2O_2$: C 62.36, H 3.66, Cl 18.41, N 7.27. Found: C 62.62, H 3.86, Cl 18.56, N 7.58%.

4.3.5. Ethyl 2-methyl-2H-benzo[g]indazole-3-carboxylate (**25**)

Yield: 95%; yellowish solid; m.p. 71–72 °C. R_f (petroleum ether/ethyl acetate 8:2) 0.62. IR (nujol): 1710 (C=O). UV (ethanol): λ_{max} 216 (3.95), 222 (3.96), 242 (3.56), 255 (3.58), 281 (3.49), 293 (3.53), 306 (3.33), 323 (3.23). ^{1}H NMR ($CDCl_3$): 1.52 (t, 3H, $J = 7.2$ Hz), 4.50 (q, 2H, $J = 7.2$ Hz), 4.53 (s, 3H), 7.50–7.68 (m, 3H), 7.80–7.95 (m, 2H), 8.55–8.65 (m, 1H). *Anal*. Calc. for $C_{15}H_{14}N_2O_2$: C 70.85, H 5.55, N 11.02. Found: C 70.99, H 5.86, N 11.38%.

4.4. General procedure for preparation of benzo[g]indazole-carboxylic acids **3**–**7** and dihydro-indeno[1,2-c]pyrazole-carboxylic acids **8**–**11**

To appropriate ester (5 mmol) [**21**–**29**] in methanol (25 ml) was added a solution of potassium hydroxide (10 mmol) in methanol (18 ml) and some drops of water. The resulting mixture was heated under reflux overnight. The mixture was allowed to cool to room temperature and then poured into water and acidified with 1 N hydrochloric acid. The precipitate was filtered, washed with water and air-dried to yield the analytically pure acid.

4.4.1. 1H-Benzo[g]indazole-3-carboxylic acid (**3**)

Yield: 88%; white solid; m.p. 300–301 °C. R_f ($CHCl_3$/MeOH 7:3) 0.30. IR (nujol): 1690 (C=O), 3100–3200 (NH), 3300–3500 (OH). UV (ethanol): λ_{max} 210 (4.22), 236 (4.45), 268 (4.17), 279 (4.03), 291 (4.01), 302 (3.66), 316 (3.71), 331 (3.73). ^{1}H NMR ($CDCl_3$): 4.87 (br s, 2H, OH and NH exch. with D_2O), 7.54–7.62 (m, 3H), 7.94 (dd, 1H, $J = 2.2$ and 6.4 Hz), 8.14 (d, 1H, $J = 9.2$ Hz), 8.53 (dd, 1H, $J = 2.0$ and 7.6 Hz). *Anal*. Calc. for $C_{12}H_8N_2O_2$: C 69.02, H 3.80, N 13.20. Found: C 68.84, H 3.52, N 13.14%.

4.4.2. 1-Methyl-1H-benzo[g]indazole-3-carboxylic acid (**4**)

Yield: 79%; white solid; m.p. 261–263 °C. R_f ($CHCl_3$/MeOH 7:3) 0.67. IR (nujol): 1680 (C=O), 3300–3500 (OH). UV (ethanol): λ_{max} 223 (4.35), 267 (3.79), 278 (3.80), 304 (3.46), 319 (3.50). ^{1}H NMR ($CDCl_3$): 4.62 (br s, 3H and OH exch. with D_2O), 7.60–7.75 (m, 3H), 8.01 (d, 1H, $J = 7.8$ Hz), 8.19 (d, 1H, $J = 8.8$ Hz), 8.49 (d, 1H, $J = 7.8$ Hz). *Anal*. Calc. for $C_{13}H_{10}N_2O_2$: C 69.02, H 4.46, N 12.38. Found: C 69.32, H 4.21, N 12.56%.

4.4.3. 2-Methyl-2H-benzo[g]indazole-3-carboxylic acid (**7**)

Yield: 94%; white solid; m.p. 238–239 °C. R_f ($CHCl_3$/MeOH 7:3) 0.61. IR (nujol): 1680 (C=O), 3300–3500 (OH). UV (ethanol): λ_{max} 215 (4.29), 221 (4.31), 246 (4.04), 288 (3.92), 306 (3.69), 321 (3.61). ^{1}H NMR ($CDCl_3$, DMSO): 4.53 (br s, 3H and OH exch. with D_2O), 7.50–7.65 (m, 3H), 7.84 (d, 1H, $J = 7.6$ Hz), 8.00 (d, 1H, $J = 9.2$ Hz), 8.57 (d, 1H, $J = 7.6$ Hz). *Anal*. Calc. for $C_{13}H_{10}N_2O_2$: C 69.02, H 4.46, N 12.38. Found: C 69.35, H 4.75, N 12.53%.

4.4.4. 1-Phenyl-1H-benzo[g]indazole-3-carboxylic acid (**5**)

Yield: 98%; white solid; m.p. 277–278 °C. R_f ($CHCl_3$/MeOH 7:3) 0.76. IR (nujol): 1690 (C=O), 3200–3500 (OH). UV (ethanol): λ_{max} 224 (3.66), 239 (3.65), 276 (3.21), 304 (2.86), 319 (2.91). ^{1}H NMR ($CDCl_3$, DMSO): 4.11 (br s, 1H, OH exch. with D_2O), 7.34–7.60 (m, 3H), 7.61–7.65 (m, 5H), 8.01 (d, 2H, $j = 7.8$ Hz), 8.27 (d, 1H, $J = 8.8$ Hz). *Anal*. Calc. for $C_{18}H_{12}N_2O_2$: C 74.99, H 4.20, N 9.72. Found: C 74.82, H 4.36, N 9.84%.

4.4.5. 1-(2,4-Dichlorophenyl)-1H-benzo[g]indazole-3-carboxylic acid (**6**)

Yield: 85%; yellowish solid; m.p. 268–269 °C. R_f ($CHCl_3$/MeOH 7:3) 0.76. IR (nujol): 1680 (C=O), 3300–3500 (OH). UV (ethanol): λ_{max} 220 (4.49), 236 (4.41), 254 (4.35), 319 (3.54), 336 (3.59). ^{1}H NMR ($CDCl_3$, DMSO): 3.46 (br s, 1H, OH exch. with D_2O), 7.28–7.45 (m, 3H), 7.52–7.62 (m, 2H), 7.72 (d, 2H, $J =$ 8.6 Hz), 8.00 (d, 1H, $J = 8.4$), 8.32 (d, 1H, $J = 8.8$ Hz). *Anal*. Calc. for $C_{18}H_{10}Cl_2N_2O_2$: C 60.53, H 2.82, Cl 19.85, N 7.84. Found: C 60.85, H 2.65, Cl 19.53, N 7.56%.

4.4.6. 1,4-Dihydro-1H-indeno[1,2-c]pyrazole-3-carboxylic acid (**8**)

Yield: 98%; white-cream solid; m.p. 317–319 °C. R_f ($CHCl_3$/MeOH 7:3) 0.24. IR (nujol): 1720 (C=O), 3380 (NH). UV (ethanol): λ_{max} 216 (4.04), 252 (3.83), 269 (3.70), 276 (3.67). ^{1}H NMR ($CDCl_3$, DMSO): 3.74 (br s, 2H and OH and NH exch. with D_2O), 7.25–7.42 (m, 2H), 7.55 (d, 1H, $J = 6.8$ Hz), 7.67 (d, 1H, $J = 6.8$ Hz). *Anal*. Calc. for $C_{11}H_8N_2O_2$: C 66.00, H 4.03, N 12.99. Found: C 66.35, H 4.23, N 12.84%.

4.4.7. 1-Methyl-1,4-dihydro-indeno[1,2-c]pyrazole-3-carboxylic acid (**9**)

Yield: 84%; yellowish solid; m.p. 198–200 °C. R_f ($CHCl_3$/MeOH 7:3) 0.58. IR (nujol): 1680 (C=O), 3330 (OH). UV (ethanol): λ_{max} 217 (4.08), 246 (4.07), 248 (4.04), 271 (3.90), 279 (3.87), 333 (3.55). ^{1}H NMR ($CDCl_3$, DMSO): 3.74 (br s, 2H and OH exch. with D_2O), 4.20 (s, 3H), 7.25–7.45 (m, 2H), 7.52–7.58 (m, 2H). *Anal*. Calc. for $C_{12}H_{10}N_2O_2$: C 67.28, H 4.71, N 13.08. Found: C 67.45, H 4.51, N 13.34%.

4.4.8. 1-Phenyl-1,4-dihydro-indeno[1,2-c]pyrazole-3-carboxylic acid (**10**)

Yield: 78%; yellowish solid; m.p. 249–250 °C. ([5]: 250 °C).

4.4.9. 1-(2,4-Dichlorophenyl)-1,4-dihydro-indeno[1,2-c]pyrazole-3-carboxylic acid (**11**)

Yield: 89%; yellowish solid; m.p. 271–272 °C. ([15]: 271–272 °C).

4.5. General procedure for preparation of 1H-Benzo[g]indazole-carboxamides **2a–j** and 1,4-dihydroindeno[1,2-c]pyrazole-carboxamides **2k–r**

A mixture of the appropriate acid (1 mmol) [**3**–**11**] and CDI (1.1 mmol) in DMF (2.5 ml) was stirred at r.t. for 3 h. The reaction mixture was added of the requisite amine [(*N*,*N*-bis(2-aminoethyl)methylamine or *N*,*N*-bis(3-aminopropyl)methylamine)] (0.5 mmol) in DMF (2 ml). The mixture was stirred at r.t. for 48 h, the solvent was then removed under reduced pressure and the residue purified as reported below to give the title compounds.

4.5.1. N3-{2-[{2-[(1H-Benzo[g]indazole-3-yl-carbonyl)amino]ethyl}(methyl)amino]ethyl}-1H-benzo[g]indazole-3-carboxamide (**2a**)

Yield: 72% as an oily residue which solidified on standing after purification by FC eluting with $CHCl_3$/CH_3OH 9/1; yellowish solid; m.p. 149–150 °C. R_f ($CHCl_3$/MeOH 9:1) 0.28. IR (nujol): 1630 (C=O), 3120–3200 (NH), 3380–3400 (NH). UV (ethanol): λ_{max} 235 (4.02), 260 (3.65), 268 (3.63), 280 (3.48), 291 (3.46), 316 (3.10), 330 (3.16). ^{1}H NMR ($CDCl_3$, DMSO): 2.42 (s, 3H), 2.75 (t, 4H), 3.55–3.72 (m, 4H), 5.50–5.90 (br s, 2H, NH exch. with D_2O), 7.45–8.45 (m, 12H and 2NH exch. with D_2O). *Anal*. Calc. for $C_{29}H_{27}N_7O_2$: C 68.69, H 5.38, N 19.39. Found: C 68.72, H 5.52, N 19.63%.

*4.5.2. N3-{3-[{3-[(1H-Benzo[g] indazole-3-yl-carbonyl)amino]propyl}(methyl)amino]propyl}-1H-benzo[g]indazole-3-carboxamide (**2b**)*

Yield: 27% after purification by trituration with acetone; white solid; m.p. 230–232 °C. R_f ($CHCl_3$/MeOH 9:1) 0.17. IR (nujol): 1640 (C=O), 3100–3160 (NH), 3360 (NH). UV (ethanol): λ_{max} 236 (4.34), 248 (4.01), 268 (3.97), 280 (3.80), 291 (3.79), 316 (3.44), 331 (3.48). ^{1}H NMR ($CDCl_3$, DMSO): 1.82–2.02 (qu., 4H), 2.29 (s, 3H), 2.57 (t, 4H), 3.10–3.40 (br s, 4H, NH exch. with D_2O), 3.50–3.70 (m, 4H), 7.45–8.65 (m, 10H and 2NH exch. with D_2O). *Anal*. Calc. for $C_{31}H_{31}N_7O_2$: C 69.77, H 5.86, N 18.37. Found: C 69.84, H 5.63, N 18.56%.

*4.5.3. N3-{2-[{2-[(1-Methyl-1H-benzo[g]indazole-3-yl-carbonyl)amino]ethyl}(methyl)amino]ethyl}-1-methyl-1H-benzo[g]indazole-3-carboxamide (**2c**)*

Yield: 70% as an oily residue which solidified on standing after purification by FC eluting with $CHCl_3$/CH_3OH 9/1; white solid; m.p. 120–122 °C (triturated with ethyl ether). R_f ($CHCl_3$/MeOH 9:1) 0.50. IR (nujol): 1650 (C=O), 3100–3400 (NH). UV (ethanol): λ_{max} 221 (4.59), 254 (4.12), 269 (4.00), 280 (4.02), 304 (3.65), 320 (3.67). ^{1}H NMR ($CDCl_3$): 2.48 (s, 3H), 2.75 (t, 4H), 3.65 (q, 4H), 3.98 (s, 6H), 7.11 (br s, 2H, NH exch. with D_2O), 7.25–8.35 (m, 1H). *Anal*. Calc. for $C_{31}H_{31}N_7O_2$: C 69.77, H 5.86, N 18.37. Found: C 69.83, H 5.67, N 18.53%.

*4.5.4. N3-{3-[{3-[(1-Methyl-1H-benzo[g] indazole-3-yl-carbonyl)amino]propyl}(methyl)amino]propyl}1-methyl-1H-benzo[g]indazole-3-carboxamide[(E)-3-carboxy-2-propenoate] (**2d**)*

Yield: 53% as an oily residue which solidified on standing after purification by FC eluting with $CHCl_3$/CH_3OH 8/2; white solid; m.p. 124–125 °C (as fumarate). R_f ($CHCl_3$/MeOH 8:2) 0.47. IR (nujol): 1650 (C=O), 3300–3500 (NH). UV (ethanol): λ_{max} 213 (4.08), 237 (4.33), 260 (3.93), 268 (3.85), 283 (2.87), 295 (3.23), 319 (3.35), 333 (3.57). ^{1}H NMR ($CDCl_3$): 1.93 (qu 4H, J = 6.4 Hz), 2.33 (s, 3H), 2.59 (t, 2H, J = 6.4 Hz), 3.64 (q, 4H, J = 6.4 Hz), 4.39 (s, 6H), 6.68 (br s, 2H, NH exch. with D_2O), 7.05–8.35 (m, 14H). *Anal*. Calc. for $C_{33}H_{35}N_7O_2 \cdot 2C_4H_4O_4$: C 61.72, H 5.94, N 12.29. Found: C 61.69, H 6.06, N 12.64%.

*4.5.5. N3-{2-[{2-[(2-Methyl-2H-benzo[g]indazole-3-yl-carbonyl)amino]ethyl}(methyl)amino]ethyl}2-methyl-2H-benzo[g]indazole-3-carboxamide[(E)-3-carboxy-2-propenoate] (**2e**)*

Yield: 42% as an oily residue which solidified on standing after purification by FC eluting with $CHCl_3$/CH_3OH 9/1; white solid; m.p. 133–134 °C (as fumarate). R_f ($CHCl_3$/MeOH 9:1) 0.51. IR (nujol): 1650 (C=O), 3260 (NH). UV (ethanol): λ_{max} 223 (4.55), 242 (4.36), 285 (4.18), 308 (3.93), 319 (3.81). ^{1}H NMR ($CDCl_3$): 2.46 (s, 3H), 2.77 (q, 4H, J = 5.6 Hz), 3.62 (q, 4H, J = 5.6 Hz), 4.13 (s, 6H), 6.70–6.85 (br s, 2H, NH exch. with D_2O), 7.10–7.18 (m, 4H), 7.40–7.60 (m, 6H), 8.25–8.35 (m, 2H). *Anal*. Calc. for $C_{31}H_{31}N_7O_2 \cdot 2C_4H_4O_4 \cdot H_2O$: C 59.46, H 5.76, N 12.45. Found: C 60.07, H 5.74, N 12.41%.

*4.5.6. N3-{3-[{3-[(2-Methyl-2H-benzo[g]indazole-3-yl-carbonyl)amino]propyl}(methyl) amino]propyl}2-methyl-2H-benzo[g]indazole-3-carboxamide (**2f**)*

Yield: 30% as an oily residue which solidified on standing after purification by FC eluting with $CHCl_3$/CH_3OH 9/1; yellowish solid; m.p. 117–118 °C (triturated with ethyl ether). R_f ($CHCl_3$/MeOH 9:1) 0.30. IR (nujol): 1660 (C=O), 3300 (NH). UV (ethanol): λ_{max} 239 (4.67), 248 (4.50), 299 (4.32). 319 (4.06), 334 (3.96). ^{1}H NMR ($CDCl_3$): 1.70–1.89 (m, 4H), 2.33 (s, 3H), 2.45–2.65 (m, 2H), 3.45–3.60 (m, 4H), 4.30–4.15 (br s, 2H, NH exch. with D_2O), 4.30 (s, 6H), 7.25–8.50 (m, 14H), 10.51 (br s, 2H, NH exch. with D_2O). *Anal*. Calc. for $C_{33}H_{35}N_7O_2$: C 70.57, H 6.28, N 17.46. Found: C 70.46, H 6.59, N 17.63%.

*4.5.7. N3-{2-[{2-[(1-Phenyl-1H-benzo[g]indazole-3-yl-carbonyl)amino]ethyl}(methyl) amino]ethyl}1-phenyl-1H-benzo[g]indazole-3-carboxamide (**2g**)*

Yield: 35% as an oily residue after purification by FC eluting with $CHCl_3$/CH_3OH 9/1; yellowish oil; m.p. 118–120 °C (as hydrochloride). R_f ($CHCl_3$/MeOH 9:1) 0.31. IR (nujol): 1630 (C=O), 3300–3400 (NH). UV (ethanol): λ_{max} 220 (4.57), 244 (4.47), 254 (4.41), 279 (4.16), 306 (3.77), 314 (3.57), 320 (3.77). ^{1}H NMR ($CDCl_3$): 2.40 (s, 3H), 2.73 (t, 4H, J = 6.4 Hz), 3.51–3.69 (m, 4H, J = 6.4 Hz), 7.20–7.50 (m, 16H and 2NH exch. with D_2O), 7.65 (d, 2H, J = 8.8 Hz), 7.88 (d, 2H, J = 8.2 Hz), 8.39 (d, 2H, J = 8.8 Hz). *Anal*. Calc. for $C_{41}H_{35}N_7O_2 \cdot 2HCl \cdot H_2O$: C 65.77, H 5.25, Cl 9.47, N 13.10. Found: C 65.87, H 5.09, Cl 9.32, N 12.81%.

*4.5.8. N3-{3-[{3-[(1-Phenyl-1H-benzo[g]indazole-3-yl-carbonyl)amino]propyl}(methyl) amino]propyl}1-phenyl-1H-benzo[g]indazole-3-carboxamide (**2h**)*

Yield: 20% as an oily residue after purification by FC eluting with $CHCl_3$/CH_3OH 9/1; yellowish oil; m.p. 124–126 °C (as hydrochloride). R_f ($CHCl_3$/MeOH 9:1) 0.41. IR (nujol): 1660 (C=O), 3300–3400 (NH). UV (ethanol): λ_{max} 212 (4.48), 223 (4.45), 242 (4.40), 253 (4.37), 273 (4.10), 307 (3.61), 320 (3.63). ^{1}H NMR ($CDCl_3$): 1.79 (qu, 4H, J = 6.4 Hz), 2.21 (s, 3H), 2.50 (t, 4H, J = 6.4 Hz), 3.46–3.58 (m, 4H, J = 6.4 Hz), 7.21–7.31 (m, 2H), 7.45–7.64 (m, 16H and 2NH exch. with D_2O), 7.83–7.95 (m, 2H), 8.42 (d, 2H,, J = 9.0 Hz). *Anal*. Calc. for $C_{43}H_{39}N_7O_2 \cdot 3HCl \cdot 3H_2O$: C 60.81, H 5.70, Cl 12.52, N 11.55. Found: C 60.54, H 5.47, Cl 12.35, N 11.25%.

*4.5.9. N3-{2-[2-{[{1-(2,4-Dichlorophenyl)-1H-benzo[g]indazole-3-yl-carbonyl}amino]ethyl}(methyl)amino]ethyl}1-(2,4-dichlorophenyl)-1H-benzo[g]indazole-3-carboxamide (**2i**)*

Yield: 28% as an oily residue after purification by FC eluting with $CHCl_3/CH_3OH$ 9/1; yellowish oil; m.p. 149–152 °C (as hydrochloride). R_f ($CHCl_3$/MeOH 9:1) 0.33. IR (nujol): 1660 (C=O), 3300–3420 (NH). UV (ethanol): λ_{max} 202 (4.82), 233 (4.62), 254 (4.53), 276 (4.26), 305 (3.87), 320 (3.89). ^{1}H NMR ($CDCl_3$): 2.40 (s, 3H), 2.70–2.77 (m, 4H, $J = 6$ Hz), 3.51–3.71 (m, 4H, $J = 6$ Hz), 7.17–7.68 (m, 16H and 2NH exch. with D_2O), 7.87–7.97 (m, 1H), 8.35–8.42 (m, 1H). *Anal*. Calc. for $C_{41}H_{31}Cl_4N_7O_2 \cdot 2HCl \cdot H_2O$: C 55.55, H 3.98, Cl 24.00, N 11.06. Found: C 55.39, H 3.64, Cl 23.98, N 11.04%.

*4.5.10. N3-{3-[3-{[{1-(2,4-Dichlorophenyl)-1H-benzo[g]indazole-3-yl-carbonyl}amino]propyl}(methyl)amino]propyl}1-(2,4-dichlorophenyl)-1H-benzo[g]indazole-3-carboxamide (**2j**)*

Yield: 52% as an oily residue after purification by FC eluting with $CHCl_3/CH_3OH$ 9/1; yellowish oil. M.p. 156–157 °C (as hydrochloride). R_f ($CHCl_3$/MeOH 9:1) 0.33. IR (nujol): 1660 (C=O), 3280–3420 (NH). UV (ethanol): λ_{max} 234 (4.88), 254 (4.80), 274 (4.53), 290 (4.02), 305 (4.18), 320 (4.23). ^{1}H NMR ($CDCl_3$): 1.70–1.95 (m, 4H, $J = 5.2$ Hz), 2.21 (s, 3H), 2.39–2.60 (t, 4H, $J = 5.2$ Hz), 3.40–3.60 (m, 4H, $J = 5.2$ Hz), 7.19–7.70 (m, 16H and 2NH exch. with D_2O), 7.87–8.07 (m, 1H), 8.32–8.43 (m, 1H). *Anal*. Calc. for $C_{45}H_{35}Cl_4N_7O_2 \cdot 2HCl \cdot 2H_2O$: C 55.38, H 4.43, Cl 22.81, N 10.51. Found C 55.35, H 4.40, Cl 22.78, N 10.48%.

*4.5.11. N3-{2-[{2-[(1,4-Dihydroindeno[1,2-c]pyrazole-3-yl-carbonyl)amino]ethyl}(methyl)amino]ethyl}-1,4-dihydroindeno[1,2-c]pyrazole-3-carboxamide (**2k**)*

Yield: 33% as an oily residue which solidified on standing after purification by FC eluting with $CHCl_3/CH_3OH$ 9/1; yellowish solid; m.p. 207–208 °C (triturated with ethyl ether). R_f ($CHCl_3$/MeOH 9:1) 0.26. IR (nujol): 1630 (C=O), 3120 (NH), 3400 (NH). UV (ethanol): λ_{max} 230 (4.00), 254 (3.91), 270 (3.74), 278 (3.69). ^{1}H NMR ($CDCl_3$): 2.37 (s, 3H), 2.65–2.72 (m, 4H), 3.49–3.60 (m, 4H), 3.71 (s, 4H), 7.18–7.65 (m, 10H and 2NH exch. with D_2O).). *Anal*. Calc. for $C_{27}H_{27}N_7O_2$: C 67.34, H 5.60, N 20.36. Found: C 67.68, H 5.62, N 20.56%.

*4.5.12. N3-{3-[{3-[(1,4-Dihydroindeno[1,2-c]pyrazole-3-yl-carbonyl)amino]propyl}(methyl)amino]propyl}-1,4-dihydroindeno[1,2-c]pyrazole-3-carboxamide (**2l**)*

Yield: 25% as an oily residue which solidified on standing after purification by FC eluting with $CHCl_3/CH_3OH$ 9/1; white solid. m.p. 166–167 °C (triturated with ethyl ether). R_f ($CHCl_3$/MeOH 9:1) 0.22. IR (nujol): 1640 (C=O), 3100–3200 (NH), 3400 (NH). UV (ethanol): λ_{max} 228 (3.68), 242 (3.66), 254 (3.60), 271 (3.40), 278 (3.35). ^{1}H NMR ($CDCl_3$, DMSO): 1.88 (qu, 4H, $J = 5.4$ Hz), 2.30 (s, 3H), 2.50–2.63 (m, 4H, $J = 5.4$ Hz), 2.64–2.78 (br s, 2H, NH exch. with D_2O), 3.51–3.69 (m, 4H, $J = 5.4$ Hz), 3.82 (s, 4H), 7.20–7.40 (m, 4H and 2NH exch. with D_2O), 7.51 (d, 2H, $J = 6.8$ Hz), 7.70 (d, 2H, $J = 6.2$ Hz). *Anal*. Calc. for $C_{29}H_{31}N_7O_2$: C 68.35, H 6.13, N 19.24. Found: C 68.47, H 6.35, N 19.52%.

*4.5.13. N3-{2-[{2-[(1-Methyl-1,4-dihydroindeno[1,2-c]pyrazole-3-yl-carbonyl)amino]ethyl}(methyl)amino]ethyl}-1-methyl-1,4-dihydroindeno[1,2-c]pyrazole-3-carboxamide (**2m**)*

Yield: 17% as an oily residue which solidified on standing after purification by FC eluting with $CHCl_3/CH_3OH$ 8/2; white-cream solid; m.p. 153–155 °C (triturated with ethyl ether). R_f ($CHCl_3$/MeOH 9:1) 0.47. IR (nujol): 1670 (C=O), 3400 (NH). UV (ethanol): λ_{max} 239 (4.36), 263 (4.39), 274 (4.37), 284 (4.18), 295 (4.08). ^{1}H NMR ($CDCl_3$): 2.41 (s, 3H), 2.69 (t, 4H, $J = 6$ Hz), 3.57 (q, 4H, $J = 6$ Hz), 3.69 (s, 4H), 3.93 (s, 6H); 7.15–7.55 (m, 8H and 2NH exch. with D_2O). *Anal*. Calc. for $C_{29}H_{31}N_7O_2$: C 68.35, H 6.13, N 19.24. Found: C 68.52, H 6.41, N 19.51%.

*4.5.14. N3-{3-[{3-[(1-Methy-1,4-dihydroindeno[1,2-c]pyrazole-3-yl-carbonyl)amino]propyl}methylamino]propyl}1-methyl-1,4-dihydroindeno[1,2-c]pyrazole-3-carboxamide[(E)-3-carboxy-2-propenoate] (**2n**)*

Yield: 62% as an oily residue after purification by FC eluting with $CHCl_3/CH_3OH$ 9/1; yellowish oil: m.p. 107–108 °C (as fumarate). R_f ($CHCl_3$/MeOH 8:2) 0.41. IR (nujol): 1670 (C=O), 3400 (NH). UV (ethanol): λ_{max} 246 (4.71), 258 (4.69), 278 (4.59). ^{1}H NMR ($CDCl_3$): 1.85 (qu, 4H, $J = 6.4$ Hz), 2.28 (s, 3H), 2.52 (t, 4H, $J = 6.4$ Hz), 3.55 (q, 4H, $J = 6.4$ Hz), 3.68 (s, 4H), 4.08 (s, 6H), 7.25–7.69 (m, 8H and 2NH exch. with D_2O). *Anal*. Calc. for $C_{31}H_{35}N_7O_2 \cdot C_4H_4O_4 \cdot H_2O$: C 62.39, H 6.43, N 14.55. Found: C 62.36, H 6.41, N 14.51%.

*4.5.15. N3-{2-[{2-[(1-Phenyl-1,4-dihydroindeno[1,2-c]pyrazole-3-yl-carbonyl)amino]ethyl}(methyl)amino]ethyl}1-phenyl-1,4-dihydroindeno[1,2-c]pyrazole-3-carboxamide (**2o**)*

Yield: 20% as an oily residue which solidified on standing after purification by FC eluting with $CHCl_3/CH_3OH$ 9/1; white solid; m.p. 135–139 °C (triturated with ethyl ether). R_f ($CHCl_3$/MeOH 9:1) 0.75. IR (nujol): 1670 (C=O), 3380 (NH). UV (ethanol): λ_{max} 256 (3.35), 275 (3.33), 298 (3.67). ^{1}H NMR ($CDCl_3$): 2.40 (s, 3H), 2.73 (t, 4H, $J = 6.2$ Hz), 3.60 (q, 4H, $J = 6.2$

Hz), 3.61 (s, 4H), 7.15–7.60 (m, 18H and 2NH exch. with D_2O). *Anal*. Calc. for $C_{39}H_{35}N_7O_2$: C 73.91, H 5.57, N 15.47. Found: C 73.85, H 5.42, N 15.23%.

4.5.16. N3-{3-[{3-[(1-Phenyl-1,4-dihydroindeno[1,2-c]pyrazole-3-yl-carbonyl)amino]propyl}(methyl)amino]propyl}1-phenyl-1,4-dihydroindeno[1,2-c]pyrazole-3-carboxamide (**2p**)

Yield: 56% as an oily residue which solidified on standing after purification by FC eluting with $CHCl_3$/CH_3OH 8/2; yellowish solid; m.p. 85–88 °C (triturated with ethyl ether). R_f ($CHCl_3$/MeOH 8:2) 0.79. IR (nujol): 1660 (C=O), 3400 (NH). UV (ethanol): λ_{max} 235 (3.79), 248 (3.81), 281 (3.63). ^1H NMR ($CDCl_3$): 1.82 (qu, 4H, $J = 6.4$ Hz), 2.28 (s, 3H), 2.52 (t, 4H, $J = 6.4$ Hz), 3.55 (q, 4H, $J = 6.4$ Hz), 3.68 (s, 4H), 7.20–7.30 (m, 4H), 7.38–7.48 (m, 2H and 2NH exch. with D_2O), 7.50–7.55 (m, 6H), 7.65–7.75 (m, 6H). *Anal*. Calc. for $C_{41}H_{39}N_7O_2$: C 74.41, H 5.94, N 14.82. Found: C 74.52, H 5.64, N 14.56%.

4.5.17. N3-{2-[{2-[{1-(2,4-Dichlorophenyl)-1,4-dihydroindeno[1,2-c]pyrazole-3-yl-carbonyl}amino]ethyl}(methyl)amino]ethyl}1-(2,4-dichlorophenyl)-1,4-dihydroindeno[1,2-c] pyrazole-3-carboxamide (**2q**)

Yield: 37% as an oily residue which solified on standing after purification by FC eluting with $CHCl_3$/CH_3OH 8/2; yellowish solid; m.p. 153–154 °C (triturated with ethyl ether). R_f ($CHCl_3$/MeOH 8:2) 0.70 IR (nujol): 1660 (C=O), 3420 (NH). UV (ethanol): λ_{max} 230 (3.92), 280 (3.72). ^1H NMR ($CDCl_3$): 2.38 (s, 3H), 2.70 (t, 4H, $J = 5.8$ Hz), 3.56 (q, 4H, $J = 5.8$ Hz), 3.84 (s, 4H), 6.96 (d, 2H, $J = 7.0$ Hz), 7.18–7.36 (m, 8H), 7.40–7.55 (m, 4H and 2NH exch. with D_2O). *Anal*. Calc. for $C_{39}H_{31}Cl_4N_7O_2$: C 60.71, H 4.05, Cl 18.38, N 12.71. Found: C 60.45, H 4.23, Cl 18.38, N 12.63%.

4.5.18. N3-{3-[{3-[{1-(2,4-Dichlorophenyl)-1,4-dihydroindeno[1,2-c]pyrazole-3-yl-carbonyl}amino]propyl}(methyl)amino]propyl}1-(2,4-dichlorophenyl)-1,4-dihydroindeno[1,2-c] pyrazole-3-carboxamide (**2r**)

Yield: 48% as an oily residue which solified on standing after purification by FC eluting with $CHCl_3$/CH_3OH 9/1; yellowish solid; m.p. 117–118 °C (triturated with ethyl ether). R_f ($CHCl_3$/MeOH 9:1) 0.36 IR (nujol): 1660 (C=O), 3300–3420 (NH). UV (ethanol): λ_{max} 232 (3.86), 263 (3.76), 272 (3.72), 281 (3.87). ^1H NMR ($CDCl_3$): 1.82 (qu, 4H, $J = 6$ Hz), 2.31 (s, 3H), 2.54 (t, 4H, $J = 6$ Hz), 3.55 (q, 4H, $J = 6$ Hz), 3.68 (s, 4H), 6.96 (d, 2H, $J = 7.0$ Hz), 7.18–7.35 (m, 4H), 7.40–7.43 (m, 2H), 7.50–7.65 (m, 6H and 2NH exch. with D_2O). *Anal*. Calc. for $C_{41}H_{35}Cl_4N_7O_2$: C 61.59, H 4.41, Cl 17.74, N 12.26. Found: C 61.87, H 4.12, Cl 17.53, N 12.45%.

4.6. Biology

4.6.1. In vitro cytotoxicity assay

The cellular response to drugs was evaluated utilizing the solforhodamine B assay as described [7,17]. Briefly, the human tumor cell lines making up the NCI cancer screening panel were routinely grown in RPMI 1640 medium containing 5% fetal bovine serum and 2 mM L-glutamine. Cells were inoculated into 96-well microtiter plates in 100 μl of complete medium at densities ranging from 5000 to 40 000 cells/well. The microtiter plates containing cells were incubated for 24 h prior to the addition of experimental drugs. Following the addition of drugs, the plates were incubated for an additional 48 h, and cells were fixed with TCA, washed, and stained with sulforhodamine B (Sigma Chemical Co., St. Louis, MO) at 0.4% (w/v) in 1% acetic acid. After washing with 1% acetic acid, the stain was solubilized with 10 mM unbuffered Tris base and the absorbance was measured on a Bio-Tek microplate reader. Dose–response parameters were calculated as reported [18].

4.6.2. Hollow fiber assay

The cell lines are cultived in RPMI-1640 containing 10% FBS and 2 mM glutamine. On the day preceding hollow fiber preparation, the cells are given a supplementation of fresh medium to maintain log phase growth. For fiber preparation, the cells are harvested by standard trypsinization technique and resuspended at the desired cell density (varies by cell line between 2 and 10×10^6 cells ml^{-1}). The cell suspension is flushed into 1 mm ID polyvinylidene hollow fibers with a molecular weight exclusion of 500 000 Da. The hollow fibers are heat-sealed at 2 cm intervals and the samples generated from these seals are placed into tissue culture medium and incubated at 37 °C in 5% CO_2 for 24–48 h prior to implantation. A total different tumor lines are prepared for each experiment so that each mouse receives 3 IP implants (1 of each tumor line) and 3 SC implants (1 of each tumor line). One the day of implantation, samples of each tumor cell line are quantitated for viable cell mass by a stable endpoint MTT assay so that time 0 cell mass is known. Thus, the cytostatic and cytocidal capacities of the test compound can be assessed. Mice are treated with experimental agents starting on day 3 or 4 following fiber implantation and continuing once daily for a total of four doses. Each agent is assessed by IP injection at two dose levels with three mice/dose/experiment. Vehicle controls consist of six mice receiving the compound diluent only. The fibers are collected from the mice on the day following the fourth compound treatment and subjected to the stable endpoint MTT assay. The optical density of each sample is determined

spectrophotometrically at 540 nm and the mean of each treatment group is calculated. The percent net cell growth in each treatment group is calculated and compared to the percent net cell growth in the vehicle treated controls. Each compound is assessed in a total of four experiments (3 cell lines/experiment × 4 experiment = 12 cell lines).

Compounds are selected for further testing (e.g. time/dose exposure studies, preliminary pharmacology studies, SC xenograft efficacy studies) on the basis of several hollow fiber assay criteria. These include: (1) a reduction in net cell growth of 50% or greater in 10 of the 48 possible test combinations (12 cell lines × 2 sites × 2 compound doses); (2) a reduction in net cell growth of 50% or greater in a minimum of 4 of the 24 distant site combinations (IP drug/SC culture); and/or (3) cell kill of 1 or more cell lines in either implant site (reduction in the viable cell mass below the level present at the start of the experiment).

To simplify evaluation, a point system has been adopted which allows rapid viewing of the activity of a given compound. For this, a value of two is assigned for each compound dose which results in a 50% or greater reduction in viable cell mass. The IP and SC samples are scored separately so that criteria (1) and (2) can be evaluated. Compounds with a combined IP + score 20, a SC score 8 or a net cell kill of one or more cell lines are referred for further studies. The maximum possible score for an agent is 96 (12 cell lines × 2 sites × 2 dose levels × 2 [score]). These criteria were statistically validated by comparing the activity outcomes of >80 randomly selected compounds in the hollow fiber assay and in xenograft testing. This comparison indicated that there was a very low probability of missing a xenograft active compound if the hollow fiber assay were used as the initial in vivo screening tool. Because of the design of the hollow fiber assay, the results of individual cell lines are not reported since the statistical power of the assay is based on the impact of compound against the entire panel of cells. In addition to the hollow fiber results, other factors (e.g. unique structure, mechanism of action, etc.) may result in referral of a compound for further studies without the compound meeting these hollow fiber assay criteria.

4.7. Viscometric measurements

Poly(dA-dT)·poly(dA-dT) were purchased from Amersham Pharmacia Biotech; Distamycin A and EtBr were purchased from Sigma-Aldrich. Compound **2f** was used as hydrochloride.

Viscometric measurements were conducted at 40 °C using a Cannon-Manning semi-micro viscometer (size 75). The solutions of Poly(dA-dT)·poly(dA-dT), Dist, EtBr and **2f** were prepared in 300 μM sodium cacodylate buffer (pH 7.0). A sample solution of 800 μl was placed in a viscometer and the relative viscosity (η/η_0) of 95 μM Poly(dA-dT)·poly(dA-dT) with respect to sodium cacodylate buffer was measured as a function of the concentration of compound. The r value is definite as molar ratio of drug to DNA base pair.

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References

[1] P. Calabresi, B.A. Chabner, Chemotherapy of neoplastic diseases, in: J.G. Hardmann, L.E. Limbird (Eds.), Goodman and Gilman's: The Pharmacological Basis of Therapeutics, McGraw-Hill Medical Publishing Division, New York, 2001, p. 1381.
[2] R.B. Silverman (Ed.), The Organic Chemistry of Drug Design and Drug Action, Academic Press, Inc., Harcourt Brace Jovanovich Publishers, San Diego, 1992, p. 220.
[3] W.A. Denny, in: W.O. Foye (Ed.), Cancer Chemotherapeutic Agents, American Chemical Society, Washington, DC, 1995, pp. 218–239.
[4] G.A. Pinna, M.A. Pirisi, G.E. Grella, L. Gherardini, J.M. Mussinu, G. Paglietti, A.M. Ferrari, G. Rastelli, Synthesis and cytotoxicity of bis(benzo[*g*]indole-3-carboxamides) and related compounds, Arch. Pharm. Pharm. Med. Chem. 334 (2001) 337–344.
[5] R.W. Hamilton, The antiarrhythmic and antinflammatory activity of a series of tricyclic pyrazoles, J. Heterocyclic Chem. 13 (1976) 545–553.
[6] A. Alberola, L.C. Bleye, A. González-Ortega, M.L. Sábada, M.C. Sañudo, Scope and limitation in the regioselective synthesis of 1,3,5-trisubstituted pyrazoles from β-amino enones and hydrazine derivatives. ^{13}C-chemical shift prediction rules for 1,3,5-trisubstituted pyrazoles, Heterocycles 55 (2) (2001) 331–351.
[7] A. Monks, D. Scudiero, P. Skehan, R. Shoemaker, K. Paull, D. Vistica, C. Hose, J. Langley, P. Cronise, A. Vaigro-Wolff, M. Gray-Goodrich, H. Campbell, J. Mayo, M. Boyd, Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines, J. Natl. Cancer Inst. 83 (1991) 757–766.
[8] K.D. Paull, R.H. Shoemaker, L. Hods, A. Monks, D.A. Scudiero, L. Rubinstein, J. Plowman, M.R. Boyd, Display and analysis of patterns of differential activity of drugs against human tumor cell lines: development of mean graph and compare algorithm, J. Natl. Cancer Inst. 81 (1989) 1088–1092.
[9] M.R. Boyd, K.D Paull, L.R. Rubinstein, in: F.A. Valeriote, T. Corbett, L. Baker (Eds.), Cytotoxic Anticancer Drugs: Models and Concept for Drug Discovery, Kluwer Academic Publishers, Amsterdam, 1992, p. 11.
[10] J. Plowman, D.J. Dykes, M. Hollingshead, L. Simpson-Herren, M.C. Alley, Human Tumor Xenograft Models in NCI Drug Development, in: B. Teicher (Ed.), Anticancer Drug Development Guide: Preclinical Screening, Clinical Trials and Approval, Humana Press Inc, Totowa, NJ, 1997, p. 101.
[11] K. Hirata, J. Araya, S. Nakaike, K. Kitamura, T. Ishida, Side chain-dependent binding of antitumor indoloquinoxaline derivatives to DNA: comparative spectroscopic and viscometric measurements, Chem. Pharm. Bull 49 (2001) 44–48.
[12] W.E. Bachmann, N.L. Wendler, Synthesis of 1-keto-$\Delta^{4a,10a}$-dodecahydrophenanthrene, *cis*-9-methyl-1-decalone and other cyclic ketones, J. Am. Chem. Soc. 68 (1946) 2580–2584.

[13] K. Von Auwers, F. Enbergs, Bestimmung der Struktur von Derivaten des Dihydronaphto-pyrazols, J. Prakt. Chem. 134 (1932) 97–104.
[14] G. Doria, A.M. Isetta, R. Ferreccio, M. Ferrari, M.C. Fornasiero, M. Tibolla, M. Rossi, O. Carlini, P. Motta, M. Magistrelli, M. Tammiso, G. D'Antuono, M. Mameli, D. Trizio, Synthesis and immunomodulating activity of condensed *N*-aryl-2-cyano-3-oxo-3-pyrazolyl-propanamides, Farmaco 46 (1991) 843–860.
[15] J.M. Mussinu, S. Ruiu, A.C. Mulè, A. Pau, M. Carai, G. Loriga, G. Murineddu, G.A. Pinna, Tricyclic pyrazoles. I. Synthesis and biological evaluation of novel 1,4-dihydroindeno[1,2-*c*]pyrazole-based ligands for CB_1 and CB_2 cannabinoid receptors, Bioorg. Med. Chem. 11 (2003) 251–263.
[16] F. Piozzi, S. Passannanti, Sintesi di derivati idro-indazolici, Gazz. Chim. Ital. 100 (1970) 639–649.
[17] G.D. Hawkins, D.J. Giesen, G.C. Lynch, C.C. Chambers, I. Rossi, J.W. Storer, D. Rinaldi, D.A. Liotard, C.J. Cramer, D.G. Truhlar, AMSOL 6.5.3. (September 1998).
[18] P. Skehan, R. Storeng, D. Scudiero, A. Monks, J. McMahon, D. Vistica, J. Warren, H. Bokesch, S. Kenney, M. Boyd, New colorimetric cytotoxicity assay for anticancer-drug screening, J. Natl. Cancer Inst. 82 (1990) 1107–1112.