Water-soluble isoindolo[2,1-*a*]quinoxalin-6-imines: *in vitro* antiproliferative activity and molecular mechanism(s) of action

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KEYWORDS Isoindolo[2,1-*a*]quinoxalin-6-imines, antitubulin agents, topoisomerase I inhibitors, G-quadruplex interaction

ABSTRACT

Water-soluble isoindoloquinoxalin (IIQ) imines and the corresponding acetates were conveniently prepared from the key intermediates 2-(2'-aminophenyl)-2*H*-isoindole-1-carbonitriles obtained by a Strecker reaction between substituted 1,2-dicarbaldehydes and 1,2-phenylenediamines. Both series were screened by the National Cancer Institute (Bethesda, MD) and showed potent antiproliferative activity against a panel of 60 human tumor cell lines. Several of the novel compounds showed GI_{50} values at a nanomolar level on the majority of the tested cell lines. Among IIQ derivatives, methoxy substituents at positions 3 and 8 or/and 9 were especially effective in impairing cell cycle progression and inducing apoptosis in cancer cells. These effects were associated to IIQ-mediated impairment of tubulin polymerization at pharmacologically significant concentrations of tested compounds. In addition, impaired DNA topoisomerase I functions and perturbation in telomere architecture were observed in cells exposed to micromolar concentrations of IIQ derivatives. The above results suggest that IIQ derivatives exhibit multi-target cytotoxic activities.

INTRODUCTION

Quinoxalines ring system and structurally related quinoxalinones are an important class of compounds whose biological and medicinal usefulness has been widely documented due to a variety of biological activities. Several quinoxalines exhibit antineoplastic activity.[1-3] For instance, 2-arylamino-3-(arylsulfonyl)quinoxalines are PI3Ka inhibitors with IC_{50} values ranging from submicromolar to nanomolar concentrations.[4] Substituted (phenoxymethyl)quinoxalinones demonstrated excellent antagonism of P-glycoprotein and multidrug resistant protein (MRP1) in drug-resistant cell lines.[5] Heterocyclic annelated quinoxalines showed interesting antitumor activity too. As an example, quinoxaline ring condensed with a pyridine moiety led to benzo[*f*]pyrido[4,3-*b*] and pyrido[3,4-*b*]quinoxaline derivatives which showed topoisomerases inhibition.[6] Additionally, fusion with an indole ring gave 5-substituted 2-bromoindolo[3,2-*b*]quinoxalines with antitumor activity in two biochemical mechanism-based screens (cdc2 kinase and cdc25 phosphatase) with IC_{50} values of 70 and 25 μM, respectively.[7]

Starting from our previous experience on polycondensed nitrogen heterocycles containing the pyrrole,[8-14] indole[15-25] or indazole[26,27] moieties, with the aim to identify novel antitumor compounds, we extended our interest to the isoindole system.[28-32] Our efforts provided derivatives of the tetracyclic ring system isoindolo[2,1-*a*]quinoxaline (IIQ) which showed potent antiproliferative activity against the NCI human tumor cell lines panel with GI_{50} values ranging from micromolar to nanomolar concentrations[33] and high activity on vinblastine and doxorubicin resistant cell lines. Flow cytometric analysis of the cell cycle after treatment with these compounds demonstrated an arrest of the cell cycle in the G2/M phase. This effect was accompanied by apoptosis of the cells, mitochondrial depolarization, generation of reactive oxygen species, and activation of caspase-3 and caspase-9. Moreover, isoindoloquinoxalines induced a clear increase in the mitotic index, inhibited microtubule assembly *in vitro*, and

interestingly also acted as topoisomerase I inhibitors. These findings prompted us to patent such a series of compounds.[34]

Due to their poor water solubility, the above compounds were difficult to handle. In the search for optimizing pharmacodynamics and pharmacokinetics, here we report the synthesis and *in vitro* antitumor activity of water soluble imine or iminium salts (type **2** and **1** respectively) derivatives of the isoindolo[2,1-*a*]quinoxaline ring system (IIQ) as well as studies directed to elucidate their mode(s) of action. Given IIQs planar structure and charged nature, besides investigating interference with tubulin assembly or topoisomerase activity, we considered DNA binding studies as another useful aspect to be dissected. In fact IIQs might well affect both canonical (duplex) and non canonical (quadruplex) DNA structures.

RESULTS AND DISCUSSION

CHEMISTRY

Isoindoloquinoxalines (IIQs) of types **1** and **2** (Table 1) were obtained from the key intermediates 2-(2'-aminophenyl)-2*H*-isoindole-1-carbonitriles **5a-q**. In particular, derivatives **5b-e,g** were previously reported by us[33] whereas compounds **5a,f,h-q** were newly prepared (40–90%) using the same Strecker reaction between substituted 1,2-dicarbaldehydes **3a-e** and 1,2-phenylenediamines **4a-f** or 3,4-diaminophenol **4g** (Scheme 1).

The commercially unavailable 1,2-dicarbaldehydes **3b,d** and 1,2-phenylenediamine **4e** were synthesized as previously reported.[35,36] 1,2-Dicarbaldehydes **3c,e** were obtained from the corresponding **6c,e** through reduction of the carboxylic group followed by oxidation of the alcoholic functionality to obtain aldehydes **8c,e**. These latter compounds were protected,

formylated and then deprotected to afford the desired 1,2-dicarbaldehydes **3c,e** in good overall yields (54-60%) (Scheme 2).

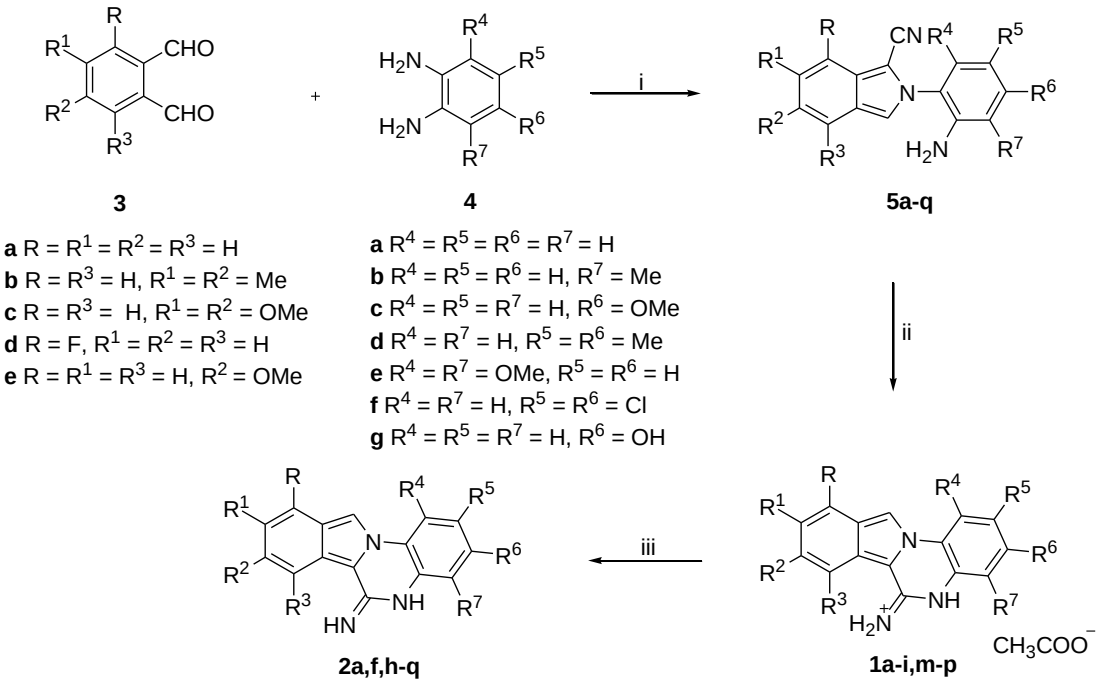
When the reaction between 1,2-dicarbaldehyde **3a** and 4-methoxy-1,2-phenylendiamine **4c** was carried at 40 °C, only isoindole **5d** was obtained because of the greater reactivity of the amino group in the *para* position than of that in the *meta* position to the methoxy substituent while at 60 °C two isoindoles **5a,d** (1/1) were formed due to the same reactivity of the two amino groups.

In the reaction between 1,2-dicarbaldehyde **3e** and 4-methoxy-1,2-phenylendiamine **4c** at 40 °C two isoindoles **5k,l** (1/1) were isolated.

Refluxing of 2-(2'-aminophenyl)-2*H*-isoindole-1-carbonitriles **5a-q** in glacial acetic acid led to IIQ-6-iminium acetates **1**. On cooling several of these salts (**1a-i,m-p**) crystallized in a pure form (55-99%) and were utilized for centesimal and spectroscopic analysis and biological tests. The remaining derivatives were neutralized with aqueous sodium hydrogen carbonate at the closely controlled temperature of -5 °C to IIQ-6-imines **2a,f,h-q** (50-99%). Iminium salts **1**, suspended in ice-water, upon neutralization as described before, gave imine derivatives **2**.

Both the iminium salts **1a,f,h-p** and the corresponding imines **2a,f,h-p** were obtained to verify their possible different behavior as antiproliferative agents.

Single crystal analysis was performed on **1d** to confirm the structure and its mesomeric form. Crystallization from acetic acid provided the specimen of **1d** on which was performed the X-ray analysis. The ORTEP representation of **1d** confirmed that the compound was indeed the acetate of the iminium derivative with the N3 bearing two hydrogen atoms and N2 bearing one hydrogen atom (according with the numbering shown in Figure 1 of SI).



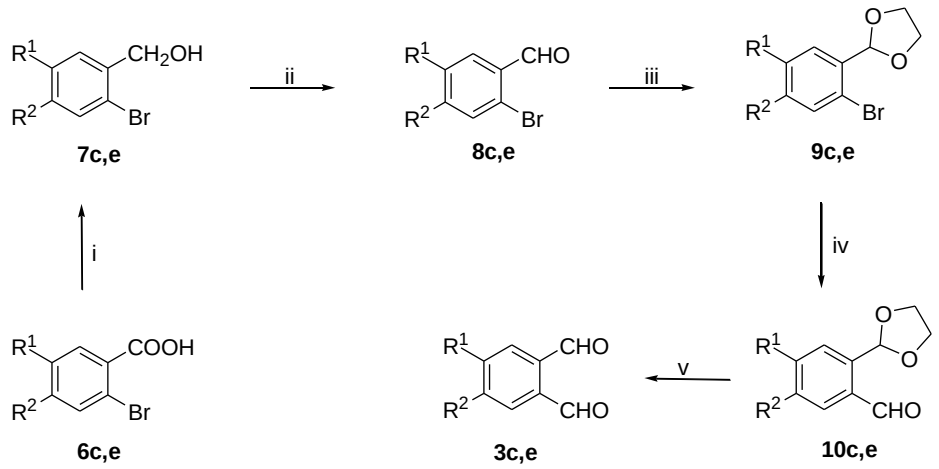
a R = R^1 = R^2 = R^3 = H
b R = R^3 = H, R^1 = R^2 = Me
c R = R^3 = H, R^1 = R^2 = OMe
d R = F, R^1 = R^2 = R^3 = H
e R = R^1 = R^3 = H, R^2 = OMe

a R^4 = R^5 = R^6 = R^7 = H
b R^4 = R^5 = R^6 = H, R^7 = Me
c R^4 = R^5 = R^7 = H, R^6 = OMe
d R^4 = R^7 = H, R^5 = R^6 = Me
e R^4 = R^7 = OMe, R^5 = R^6 = H
f R^4 = R^7 = H, R^5 = R^6 = Cl
g R^4 = R^5 = R^7 = H, R^6 = OH

Scheme 1. Synthesis of substituted isoindolo[2,1-*a*]quinoxalin-6(5*H*)-iminium acetates **1a-i**,**m-p** and isoindolo[2,1-*a*]quinoxalin-6(5*H*)-imines **2a,f,h-q**. Reagents and conditions: (i) (a) $NaHSO_3/H_2O$, rt for 3-24 h or 40-60 °C for 30 min; (b) KCN/H_2O, rt for 24 h or 40-60 °C for 90 min, 40-90%; (ii) AcOH, reflux, 30 min, 55-99%; (iii) $NaHCO_3/H_2O$, -5 °C, 50-99%.

Once the structure was confirmed, a search at the Cambridge Crystallographyc Data Base to identify structures related to **1d** was performed. Structures coded DUJMIE[37] and OSUHEQ[38] reported in Figure 2 of SI showed N3-C3 and C3-N2 distances close to those measured for **1d**. In particular, **1d** has a N3-C3 distance of 1.327 Å which is shorter than the corresponding distance in DUJMIE (1.352 Å) and longer than that of OSUHEQ (1.325 Å); Instead, C3-N2 distance in **1d** was 1.336 Å which resulted longer than that measured in DUJMIE (1.295 Å) and shorter than that found in OSUHEQ (1.351 Å). Thus, it can be argued that in the structure of **1d** there is an extensive mesomery with preference towards the exocyclic double bond N3-C3. Moreover, the only compound having the same backbone than IIQs, LIXZOH[39] (Figure 2 of SI), in which the

exocyclic nitrogen was replaced by a carbon, shows the six bond lengths of the pyrazine moiety very close to those of **1d**, and the exocyclic C-C bond (1.445 Å) shorter than a typical C-C single bond (1.500 Å) (see Table 1 of SI). The extensive mesomery in **1d** is also confirmed by the planarity of the molecule around C3.



Scheme 2. Synthesis of benzene-1,2-dicarbaldehydes **3c,e**. Reagents and conditions: (i) (a) BH_3/THF, rt, 3.5 h, (b) HCl 1 M, 0 °C, 90% for **3c** and 99% for **3e**; (ii) Dess-Martin periodinane, DCM, rt, 2 h, 90% for **3c** and 98% for **3e**; (iii) $HO(CH_2)_2OH$, *p*TsOH, PhMe, reflux, 24 h, 90%; (iv) (a) *n*BuLi, THF, -78 °C, 20-60 min, (b) DMF, 0 °C-rt, 1-5 h, 99% for **3c** and 86% for **3e**; (v) *p*TsOH, acetone, rt, 15 min, 88% for **3c** and 80% for **3e**.

Table 1. Isoindolo[2,1-*a*]quinoxalin-6(5H)-iminium acetates **1a-i,m-p** and isoindolo[2,1-*a*]quinoxalin-6(5H)-imines **2a,f,h-q** from isoindoles **5a-q**.

Compd.	R	R^1	R^2	R^3	R^4	R^5	R^6	R^7	Yield

1a	H	H	H	H	H	OMe	H	H	99%
1b	H	H	H	H	H	H	H	H	99%
1c	H	H	H	H	H	H	H	Me	95%
1d	H	H	H	H	H	H	OMe	H	99%
1e	H	H	H	H	H	Me	Me	H	90%
1f	H	H	H	H	OMe	H	H	OMe	90%
1g	H	H	H	H	H	Cl	Cl	H	99%
1h	H	Me	Me	H	H	H	OMe	H	95%
1i	H	OMe	OMe	H	H	H	OMe	H	99%
1m	H	Me	Me	H	H	H	OH	H	55%
1n	H	OMe	OMe	H	H	H	OH	H	99%
1o	F	H	H	H	H	H	OH	H	86%
1p	H	OMe	H	H	H	H	OH	H	85%
2a	H	H	H	H	H	OMe	H	H	90%
2f	H	H	H	H	OMe	H	H	OMe	90%
2h	H	Me	Me	H	H	H	OMe	H	60%
2i	H	OMe	OMe	H	H	H	OMe	H	72%
2j	F	H	H	H	H	H	OMe	H	58%

2k	H	OMe	H	H	H	H	OMe	H	60%
2l	H	H	OMe	H	H	H	OMe	H	60%
2m	H	Me	Me	H	H	H	OH	H	88%
2n	H	OMe	OMe	H	H	H	OH	H	50%
2o	F	H	H	H	H	H	OH	H	87%
2p	H	OMe	H	H	H	H	OH	H	83%
2q	H	H	OMe	H	H	H	OH	H	89%

Water solubility of the test IIQs

As expected, the novel IIQs were characterized by a remarkably improved solubility in aqueous media. A comparison of key derivatives with ISQ3 (see Figure 3 of SI), the most active of the previously reported isoindoloquinoxalines not bearing the imine function is reported in Table 2.

Table 2. Solubility in water of compounds **1i, m-p** and **2a, f, h-q** and **ISQ3**[a]

Compound	**Solubility (mM)**	**Compounds**	**Solubility (mM)**
1i	**7.2**	**2j**	**14.4**
1m	**9.8**	**2k**	**13.0**
1n	**14.0**	**2l**	**13.2**
1o	**12.7**	**2m**	**14.1**
1p	**10.9**	**2n**	**24.5**
2a	**13.8**	**2o**	**23.0**

2f	**12.7**	**2p**	**21.9**
2h	**7.8**	**2q**	**22.4**
2i	**14.4**	**ISQ3**	**3.4**

[a] Water solubility was determined by shaking an excess solute in water at 25 °C. The suspension was sonicated for 10 min, centrifuged at 8512 x g for 7 min, filtered on 0.45 μm cellulose membrane. Compound amount in saturated solution was evaluated by HPLC analysis.

Antiproliferative activity of IIQ derivatives.

All the synthesized IIQ-imines and their acetate salts were submitted to the National Cancer Institute (Bethesda MD) and prescreened, at one dose concentration (10^{-5} M), in a panel of 60 cell lines of different human tumor types. Compounds **1i**, **1m-p** and **2a**, **2f**, **2h-q** were further selected for full evaluation at five concentration levels (10^{-4}-10^{-8}M). The growth inhibition activity of compounds was defined in terms of the pGI_{50} value (which represents the −log of the molar concentration of the compound that inhibits 50% net cell growth, GI_{50}). Data reported in Table 3 revealed that the majority of derivatives of both series exhibited significant antiproliferative activity against all tested cell lines.

The most potent compounds were **2k**, **2i**, **2h** and **2l**, bearing a methoxy group in position 3. As in the preceding series, moving the methoxy group from the 3-position to 2-position decreases the activity, even if in this series in compound **2a** also the lack of the methoxy group in position 9

Table 3. Overview of the *in vitro* antitumor screening[a] results for **1i, m-p and 2a, f, h-q**

			pGI_{50}[b]	
Compd.	**N[c]**	**N[d]**	**Range**	**MG_MID**

1i	58	43	>8.00-5.02	6.45
1m	60	60	>8.00-5.28	7.08
1n	58	58	>8.00-5.48	6.34
1o	60	60	6.91-5.20	5.81
1p	60	60	>8.00-5.08	6.39
2a	46	46	6.86-5.81	6.38
2f	59	58	7.14-5.64	5.91
2h	58	56	>8.00-5.42	7.57
2i	59	56	>8.00-4.81	7.75
2j	58	54	7.68-4.97	6.95
2k	58	54	>8.00-5.92	7.87
2l	58	56	>8.00-5.98	7.70
2m	60	59	>8.00-5.84	7.20
2n	60	57	>8.00-5.49	7.45
2o	60	53	6.56-4.50	5.61
2p	59	58	7.49-4.96	6.63
2q	60	59	7.57-4.91	6.75

[a] Data obtained from the NCI in vitro disease-oriented human tumor cell line screen. [b] pGI_{50}: -log *c*, for *c* = concentration (molar) that inhibit 50% net cell growth. [c] Number of cell lines investigated. [d] Number of cell lines giving positive pGI_{50} values. [e] MG_MID: mean graph midpoint; this is the arithmetic mean value for all tested cancer cell lines. If the indicated effect was not attainable under the concentration range used, the highest tested concentration was used for the calculation.

contributes to a further decrease of the activity. In addition, the replacement of the methoxy moiety with an hydroxyl group in position 3 led to a reduction of the cytotoxic activity (compare

the pairs **2h**-**2m**, **2i**-**2n**, **2j**-**2o**, **2k**-**2p**, **2l**-**2q**). The substitution of the position 9 and/or 8 of methoxy with methyl groups slightly reduced activity (compare **2k**, **2i**, and **2h**), whereas the introduction of a fluoro at position 10 markedly reduced the biological response of the compound (compare **2k** and **2j**). In addition, a methyl or chloro substituent in position 3 led to essentially inactive compounds (compare **1e** and **1g**).

To fully describe the potential pharmacological profile of the IIQs, the outcome on cellular systems was investigated using a selection of derivatives. In this work we identify the most active compound **2k,** which did not show particular selectivity against any of the tumor sub-panels (data not shown), and derivatives **2n** and **1n** as suitable representatives of the imine and iminium series with a 3-OH substitution pattern.

IIQ derivatives cause changes in cell-cycle phase distribution and induce apoptosis.

The biological effects exerted by selected compounds (**2k**, **2n** and **1n**) were thus evaluated on human U2OS osteosarcoma cells, against which they exhibit prominent cytotoxicity at nanomolar range. The exposure of U2OS cells to IIQs resulted in a marked change in the distribution of the cells in the different phases of the cell cycle (Figure 1A), as assessed by flow cytometric analysis. Specifically, treatment (IC_{50}) of asynchronously growing U2OS cells with **2k**, **2n** and **1n** resulted in a time-dependent accumulation of cells in the G2/M phase, with a concomitant increase in the sub-G1 apoptotic cell population (Figure 1A).

In addition, at the molecular level, treatment of U2OS cells with all derivatives resulted in a marked and significant increase in caspase-3 catalytic activity, which was 13-, 23-, and 17-fold higher in cells treated for 72 h with **2k**, **2n** and **1n**, respectively, than in control cells (Figure 1B). The data are comparable to those obtained in the same cell line exposed to taxol and vinorelbine,

used as reference compounds, though a more pronounced increase in the catalytic activity of caspase-3 was appreciable after a 72-h exposure of cells to the taxane (Figure 1B).

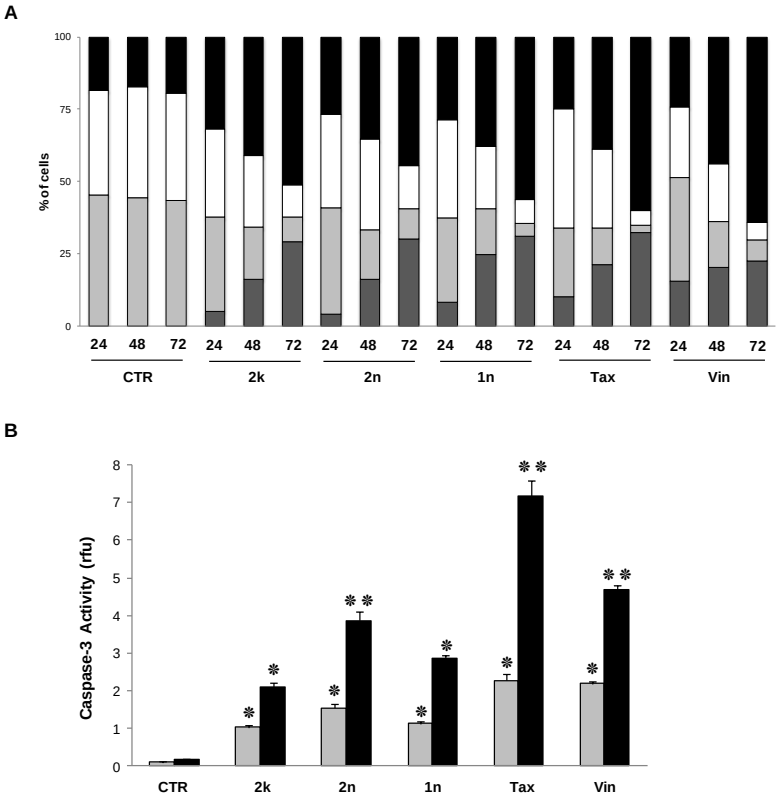


Figure 1. Effects of IIQ derivatives, taxol and vinorelbine on cell cycle phase distribution and induction of apoptosis. (A) The distribution of cells in the different phases of the cell-cycle was assessed in U2OS cells exposed for 24, 48 and 72 h to 1% DMSO (v/v) (control cells; CTR), or to the different compounds at the concentrations corresponding to the IC_{50} at 72h (**2k**, 36 nM; **2n**, 37 nM; **1n**, 23 nM; taxol, Tax, 40 nM, and Vinorelbine, Vin, 4.8 nM). The percentage of cells in sub-G1 (dark grey), G1 (light grey), S (white), and G2/M (black) phases are shown. Columns represent the mean of three independent experiments; SDs are always within 5%. (B) Caspase-3 catalytic activity was evaluated in U2OS cells exposed for 48 h (grey columns) and 72 h (black columns) to 1% (v/v) DMSO (CTR) or to **2k**, **2n**, **1n**, taxol (Tax) or vinorelbine (Vin)

(IC_{50}). Data are expressed as relative fluorescence units (rfu) and represent the mean values±SD of at least three independent experiments. $^{*}P<0.01$ and $^{**}P<0.001$, Student's t test.

Effects of IIQ derivatives on tubulin polymerization.

Based on the evidence that several derivatives structurally related to our novel IIQs were previously reported to interfere with tubulin polymerization resulting in cell-cycle arrest and induction of apoptosis, we investigated by Western blot analysis[40,41] whether this novel family of IIQs was able to display this type of activity. Results showed that all tested derivatives affected tubulin polymerization as evidenced by the reduction in the polymerized compared to soluble fraction of tubulin (Figure 2). This finding indicates that IIQ derivatives act like Vinca alkaloids, their effect on tubulin polymerization being similar to that observed in cells exposed to vinorelbine compared to cells exposed to taxol (Figure 2).

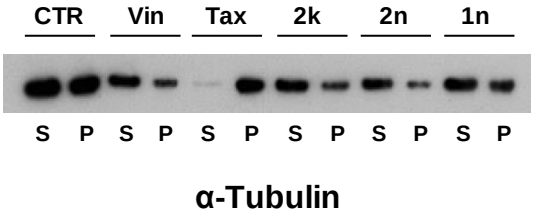


Figure 2. Effects of IIQ on tubulin polymerization. Representative western blot showing the soluble (S) or polymerized (P) tubulin fraction in U2OS human osteosarcoma cells after 24 h of exposure to different compounds at the concentrations corresponding to the IC_{50} at 72h (**2k**, 36 nM; **2n**, 37 nM; **1n**, 23 nM). Vinorelbine (Vin, 4.8 nM) and taxol (Tax, 40 nM) were selected as controls due to their opposite mechanism of action on tubulin polymerization.

Topoisomerase I-dependent DNA cleavage assay.

Topoisomerase I-mediated DNA cleavage experiments were performed to investigate the ability of IIQ to interfere with the enzyme's activity. To this purpose, purified human DNA topoisomerase I was employed and SN38, an analogue of Topotecan, was used as reference. The tested IIQ derivatives showed a topoisomerase I inhibitory activity, as suggested by the DNA cleavage pattern which was identical to that observed in samples treated with SN38. All derivatives were less potent with respect to SN38. A clear dose dependent DNA damage was observed for **2k** and **2o**, whereas a reduced potency was evidenced for high dose exposure with **2n** and **1n** (Figure 3).

The latter results can be explained taking into account potential interactions of the tested derivatives with DNA. Indeed, reduction of enzyme-mediated DNA cleavage is observed for several known DNA-binding topoisomerase poisons upon raising drug concentration due to template occupancy.[42]

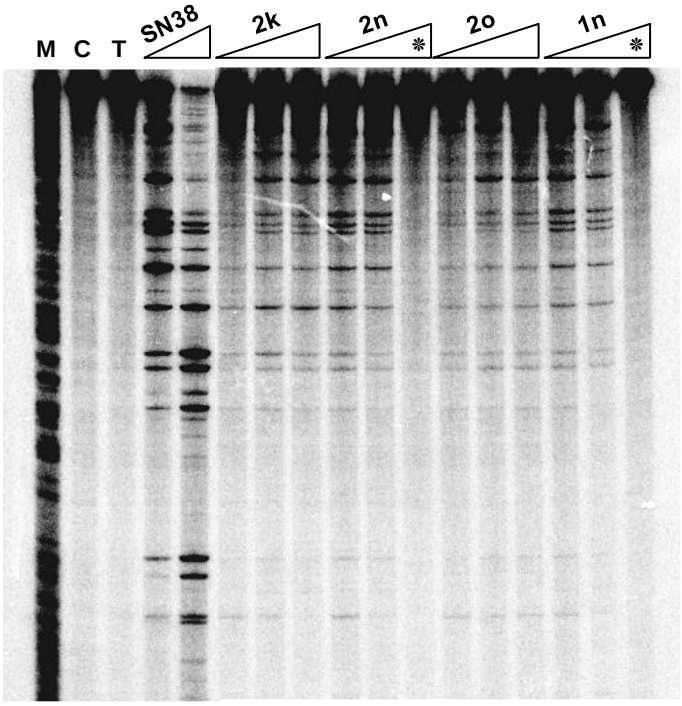


Figure 3. Topoisomerase I-mediated DNA cleavage by SN38 and IIQ derivatives. Samples were reacted with 1, 10 and 50 μM IIQ derivatives or 1 and 50 μM SN38 at 37°C for 30 min. Reaction was then stopped by adding 0.5% SDS, 0.3 mg/ml of proteinase K and incubating for 45 min at 42°C before loading on a denaturing 8% polyacrylamide gel. Asterisk indicates the reduced intensity of the DNA damage. M, purine markers; C, control DNA; T, reaction without drug.

Interaction with DNA.

DNA binding by isoindoloquinoxalin derivatives could additionally contribute to their cytotoxic activity. In fact they comprise a polycyclic system, which can be eventually protonated, creating the conditions for stacking and electrostatic binding. Thus, in order to assess whether the new compounds were actually able to interact with DNA, we investigated whether the IIQs induce a shift of the melting temperature of the nucleic acid. As DNA templates we used a random double helix fragment (duplex) and two G-rich sequences known to fold into G-

quadruplex, a human telomeric sequence (HTS) and the c-myc promorter oncogenic region (Pu24). The first represents canonically paired genomic regions, while HTS and c-myc mimic conformational situations occurring at the tips of chromosomes and at gene promotorial regions. These latter are known to arrange into differently folded G-quadruplex forms, thus providing hints about drug selectivity for a specific conformation.

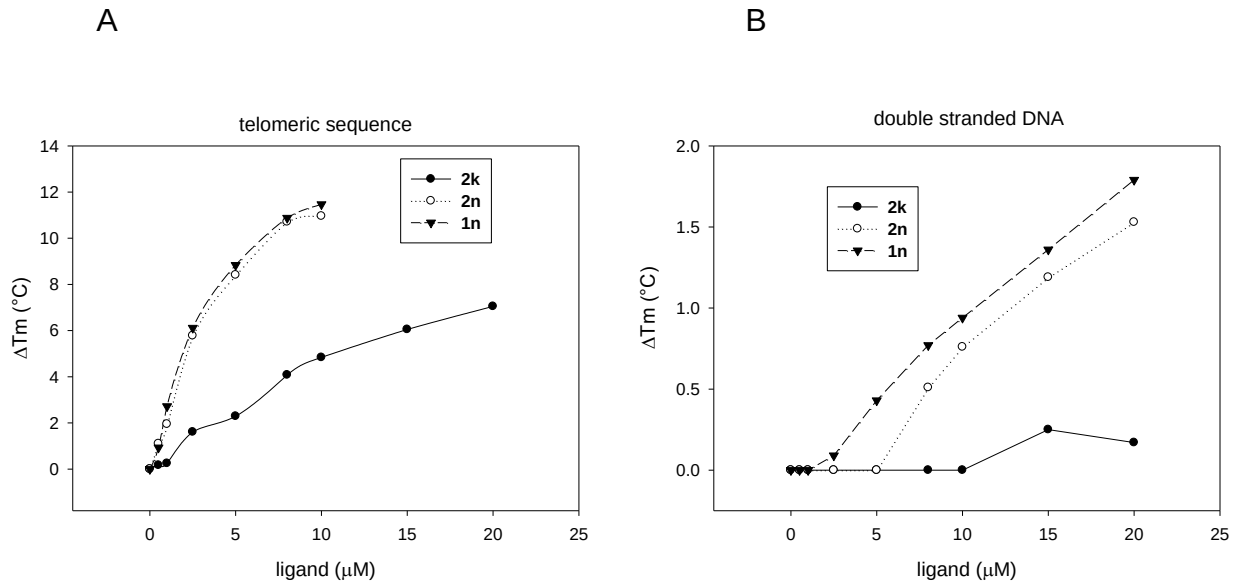


Figure 4. Variation of DNA (0.25 μM) thermal stability (ΔTm °C) produced by increasing concentration of tested ligands in 50 mM potassium buffer, pH 7.4, evaluated by fluorescence quenching melting experiments. Panel A:HTS, Panel B:double stranded DNA

Data summarized in Figure 4 underline a variable attitude of tested derivatives to stabilize the target sequences. In general, modest ΔTm were observed in the low ligand micromolar range using either double stranded or G-quadruplex DNA conformation. In fact strong binders are expected to exhibit more prominent effects.[43] However, nucleic acid stabilizing effects paralleled

biological responses. All IIQs stabilized the G-quadruplex structure of HTS more effectively than the double helix, with derivatives **1n** and **2n** being more effective than **2k**. When the screening was extended to a larger number of derivatives (Table 2 SI), the comparable activity of series **1** and **2** was confirmed with derivatives **f**, **n** and **p** that resulted the most effective in stabilizing the G-quadruplex form of HTS. Interestingly, thermal stabilization was less pronounced when the Pu24 G-quadruplex folded sequence was used, which suggests a selective interaction with the telomeric region, as compared to the promotorial region, in line with the polymorphic nature of G-quadruplex structures.

This finding gains support by the evidence that the addition of **1n** and **2n** to the telomeric sequence Tel22 caused a significant modification of the dichroic signal in the UV region (Figure 5). In particular, an increment of the intensity of the DNA dichroic band at 290 nm was always recorded. In agreement with thermal stabilization results, **2k** showed a reduced efficiency in comparison to **1n** and **2n**.

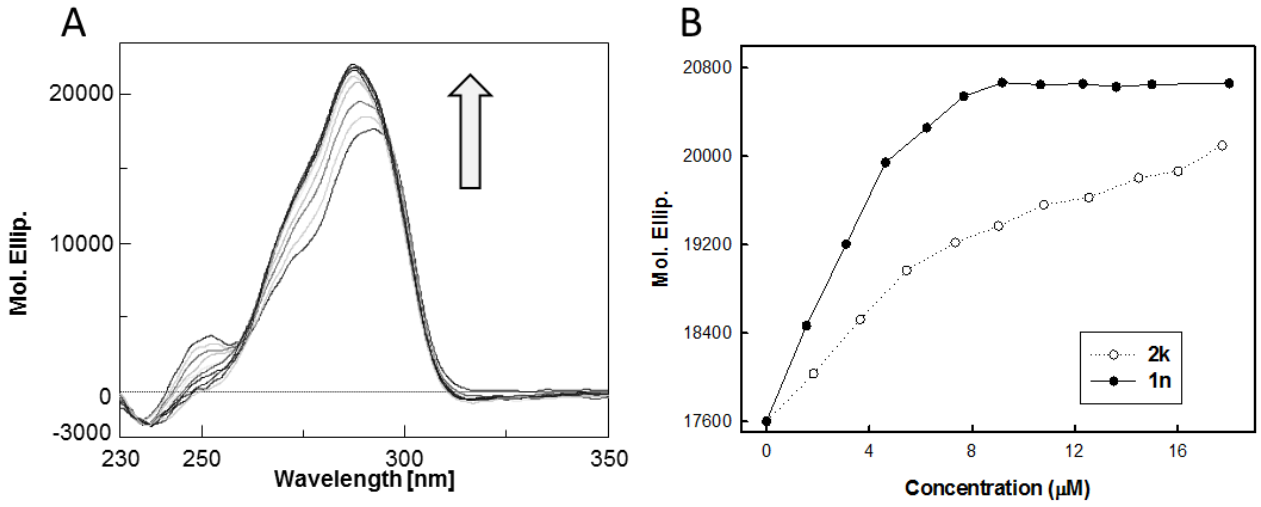


Figure 5. CD spectra of G-quadruplex folded human telomeric sequence Tel22 (4 μM) in the presence of increasing concentration of **1n** (0-18 μM) in 10 mM Tris, 50 mM KCl, pH 7.4. Arrows indicate the direction of spectral changes upon ligand addition.

Interesting results were obtained using double-stranded calf thymus DNA. In this case, the poorer ligand **2k** did not alter significantly the chiroptical response characteristic of the double helix. On the opposite, the two 8,9-dimethoxy- substituted derivatives **2n** and **1n** caused a remarkable change in the dichroic signature. In particular the positive band located at 275 nm was progressively converted into a negative one (Figure 6).

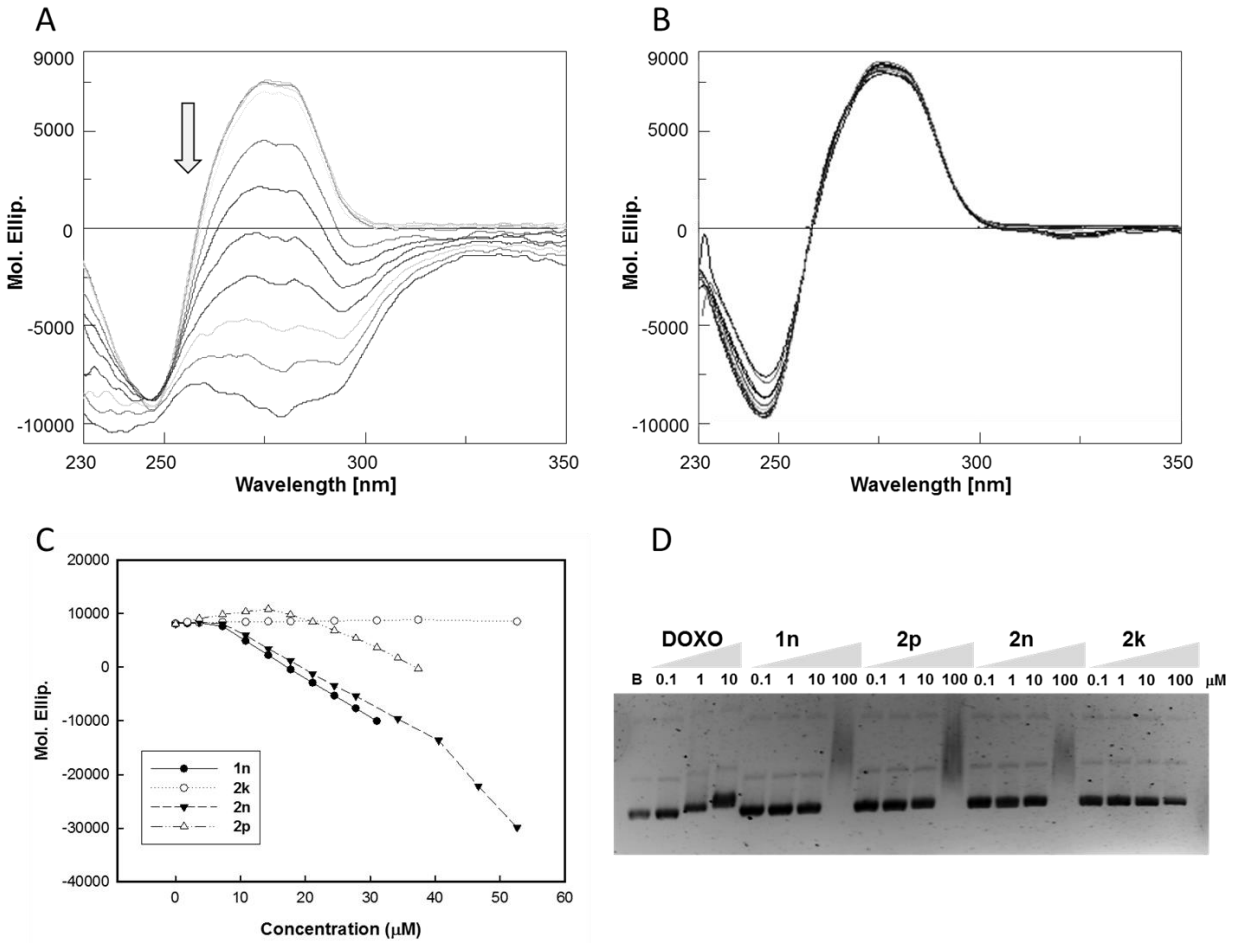


Figure 6. CD spectra of ctDNA (80 μM) in the presence of increasing concentration of **1n** (A) or **2k** (B) in 10 mM Tris, 50 mM KCl, pH 7.4. Arrow indicates spectral changes upon ligand addition. (C) Plot of the dichroic signals recorded at 275 nm as a function of ligands concentrations added to ctDNA. (D) Agarose gel of pBR322 (0.1 μg) incubated with increasing IIQs concentration.

This result indicates that upon interaction with **1n** and **2n** the DNA double helix undergoes a structural rearrangement. Although no definitive conclusion can be drawn from changes in CD pattern only, the final spectra suggest that the nucleic acid is (at least partially) converted into a psi-like form which generally reflects the onset of chiral condensates.[44] Consistently, the supercoiled plasmid pBR322 in the presence of the ligands is converted into poorly resolved slow migrating species promoted by the tested ligands. Derivative **2k** is not causing such an effect (Figure 6). This behaviour is clearly distinct from the plasmid unwinding process induced by a classical intercalative agent like doxorubicin which produced discrete bands.

Interestingly, DNA condensation processes similar to those reported above, have shown to produce important antiproliferative effects in tumour cells.[45]

In line with the enzymatic results obtained monitoring Topo I cleavage activity, the 3-hydroxy isoquinoxaline imine-DNA complex should not represent an effective substrate for enzymes devoted to process a double helix at concentration at which template occupancy prevails.

In conclusion, within the IIQ family two moderate types of DNA binding were observed, one involving the classical duplex and the other the G-rich folded structures. The binding to G-rich sequences and the possible G-quadruplex stabilizing activity of IIQs was also supported by the evidence that the derivatives were able to interfere with the arrangement of telomere-associated proteins bound to telomeric sequences in U2OS cells, as assessed by ChIP experiments (Figure

7A). Specifically, **2n** and **1n** but not **2k** induced a pronounced displacement of hPOT1 from telomeres compared to untreated cells. Such an effect was already appreciable at 6 h from the beginning of treatment, resulted in a major displacement after a 12-h exposure (-88% and -63% for **2n** and **1n**, respectively), and was followed by a complete (**2n**) or partial (-31%, **1n**) recovery of the protein binding after 24 h (Figure 7B). Conversely, the binding of TRF2 to telomeres was significantly impaired only after a 24-h exposure to all tested compounds (-67%, -80% and -66%, for **2k**, **2n** and **1n**, respectively, Figure 7B). These findings are in trend with data reported thus far. Indeed, several small molecules belonging to a variety of chemical classes (e.g., anthracene derivatives, pentacyclic acridines) have been described as selective telomeric G4-interacting agents.[46] Specifically, other than sharing common features, such as the presence of a flat aromatic surface, of cationic charges as well as the ability to stack on or intercalate in targeted G4s, these agents may trigger telomere uncapping, as a consequence of their ability to induce rapid delocalization, though with different kinetics, of telomere-associated proteins (e.g., hPOT1 and TRF2) from telomeres.[47,48]

On the other hand, in comparison to untreated cells, the binding of DNA topoisomerase I to telomeric sequences was markedly enhanced in cells exposed for 6 h (+64%) and 12 h (+309%) to **2n** and, even if to a lesser extent, in cells exposed for 12 h (+77%) to **1n** (Figure 7B). Similarly to what observed for TRF2, after long time exposure (24 h) a marked displacement of the enzyme (ranging from -57 to -78% with respect to untreated cells) was observed in osteosarcoma cells, independently of the tested derivative (Figure 7B). This evidence corroborates previous findings showing that topoisomerase I plays a role in G4 formation[49] and that its binding to telomeric sequences is enhanced in cells exposed to different G4-stabilazing agents, including RHPS4, Coron and Pip-Piper.[50]

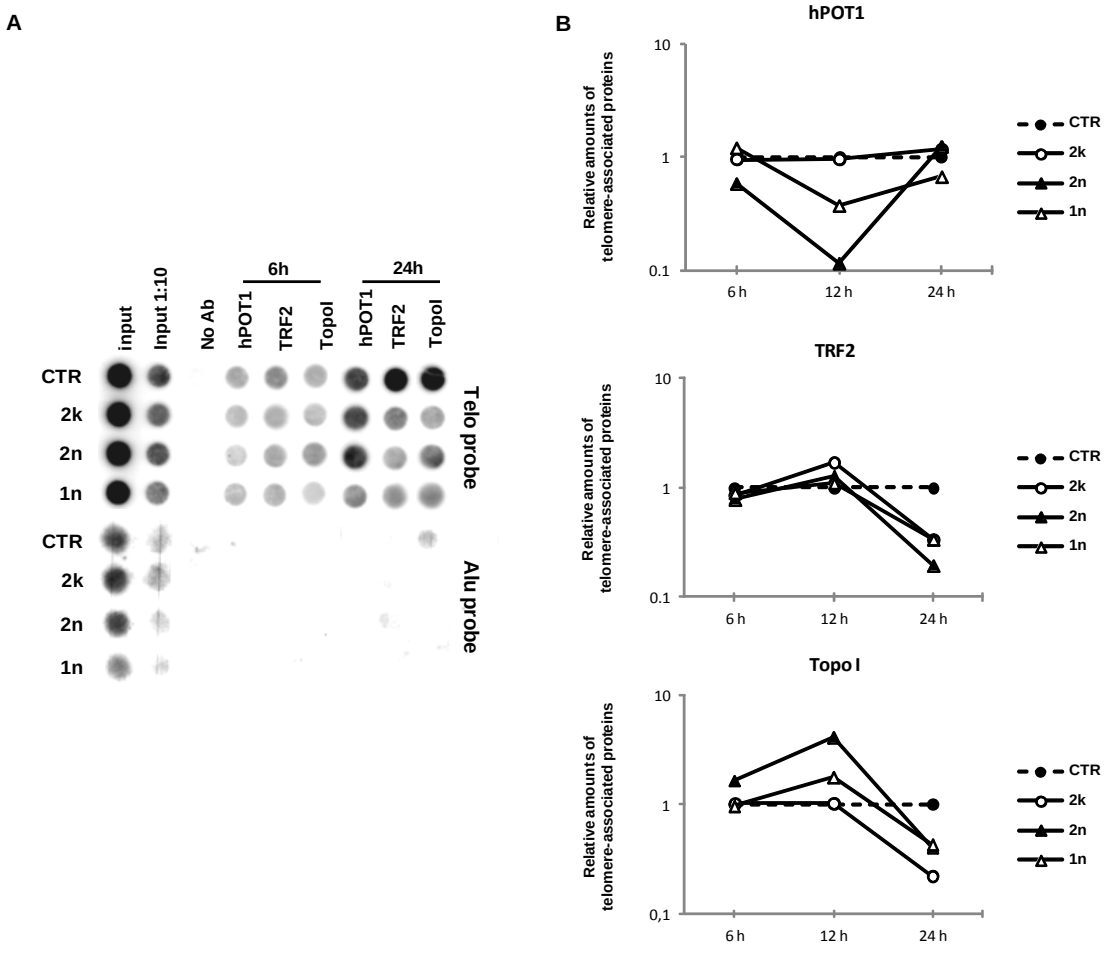


Figure 7. (A) Representative ChIP experiment showing the amount of telomere-associated proteins bound to telomeric sequences in untreated (CTR) or IIQ-treated U2OS cells. Twenty-four hours after seeding, cells were exposed to different compounds at the concentrations corresponding to the IC_{50} at 72h (**2k**, 36 nM; **2n**, 37 nM; **1n**, 23 nM). Telo probe: telomeric TTAGGG sequence used for hybridization; Alu probe: probe bearing an Alu sequence used as negative control for hybridization; Input: total chromatin used as a control for equal loading. (B)

Quantification of data from time-course ChIP experiments. Data are reported as relative amount of telomere-associated proteins bound to telomeric sequences in treated compared to untreated (control) cells. Data represents mean values from at least three independent experiments.

CONCLUSIONS

The synthesis of the novel IIQs enabled obtaining drug-like compounds endowed with appreciable solubility in aqueous media through the introduction of a protonable imino moiety. The presence of the latter group did not interfere with the cytotoxic properties of the IIQ system since the best performing compounds were active in the nanomolar range when tested against the 60 human tumor cell lines panel by NCI. In particular, the presence of methoxy groups at positions 3, 8 and/or 9 granted maximal response. Hydroxyl substitution at position 3, although reducing average response, was still compatible with significant antiproliferative properties. The two series of derivatives (**1** and **2**) behaved similarly as it could be inferred by the onset of imine protonation/deprotonation equilibria in cell systems. It should be additionally recalled that compounds with 3-hydroxyl phenolic substituents can produce zwitterionic species with the imine, leading to a differentiation in biological response (uptake, interactions with target macromolecules).

As far as mechanism of action is concerned, three of the most active compounds (**2k**, **1n**, **2n**) were investigated in detail. The tested derivatives efficiently interfere with cell cycle progression, induce an apoptotic response, and impair microtubule assembly during mitosis. Given the common and effective interference with mitotic spindle formation at nanomolar level, which closely parallels the IC_{50} trend, we infer that this is the primary mechanism of drug action.

However, additional mechanisms are apparently operating in the test derivatives. In fact, **2k** is able to produce DNA damage via Topoisomerase I poisoning and this might well add to its cytotoxicity profile. Interestingly, **2n** and **1n** are more effective in producing enzyme-mediated nucleic acid cleavage and tend to form complexes with DNA both in the double helix and in the G-quadruplex structure. As a consequence, we observe impairment of telomeric assembly and of duplex processing at cellular level. Note that, unlike **2k**, the **1n** and **2n** species bear a phenolic -OH at position 3, which can participate in hydrogen bonding to the polar nucleic acid.

Hence, the 3- methoxy derivative appears to rely more on tubulin interference than on nucleic-acid related cell killing processes, whereas a higher contribution of the latter is apparent in the 3-hydroxyl congeners.

In conclusion, at least three cytotoxicity pathways, namely inhibition of mitotic spindle formation, topoisomerase I poisoning and interference at the telomere level are likely operative in imino IIQs, the relative contributions being finely modulated by the substitution pattern and protonation state of each compound. This might explain why SAR studies are intricate in this family of drugs and the consequences of structural modifications not explainable in a straightforward manner. Hence, imino IIQs can be considered as very effective multitargeting agents endowed with drug-like physico-chemical properties, in particular adequate solubility in aqueous media, which makes them promising in view of their application in anticancer chemotherapy.

EXPERIMENTAL SECTION

CHEMISTRY

General Methods

All melting point were taken on a Büchi-Tottoly capillary apparatus and are uncorrected. IR spectra were determined in bromoform with a Jasco FT/IR 5300 spectrophotometer. ^{1}H and ^{13}C NMR spectra were measured at 200 and 50.0 MHz, respectively, in DMSO-d_6 or $CDCl_3$ solution, using a Bruker Avance II series 200 MHz spectrometer. Compounds **2a,f,h-q** were characterized only by ^{1}H NMR spectra, for their poor solubility the ^{13}C spectra were not perfomed. Column chromatography was perfomed with Merck silica gel 230-400 mesh ASTM or with Büchi Sepacor chromatography module (prepacked cartridge system). Elemental analyses (C, H, N) were within ± 0.4% of theoretical values and were performed with a VARIO EL III elemental analyzer. Both purity of all the tested compounds, which was >95%, and water solubility of compounds **1** and **2** were determined by HPLC (Agilent 1100 Series).

General procedure for the synthesis of (2-bromo-phenyl)methanols (7c,e).[51] For compounds **7c, e** see the supporting information.

General procedure for the synthesis of 2-bromo-benzaldehydes (8c,e). Dess-Martin periodinane (2.4 mmol, 1.0 g) was added to a stirred solution of the appropriate (2-bromo-phenyl)methanol **7c,e** (1.7 mmol) in dichloromethane (50 mL) under an argon atmosphere at room temperature. After 2 h, diethyl ether (50 mL) was added and the organic phase was washed successively with a 5% solution of sodium thiosulfate (2 x 50 mL) and then with a saturated solution of sodium hydrogen carbonate (3 x 80 mL). The organic phase was dried (Na_2SO_4), evaporated off under reduced pressure and the residue was purified in a column using dichloromethane as eluent. For compounds **8c,e** see supporting information.

General procedure for the synthesis of 2-(2-bromo-phenyl)1,3-dioxolanes (9c,e). To a solution of suitable 2-bromo-benzaldehyde **8c,e** (3.3 mmol) in toluene (20 mL), ethyl glicole (9.2 mmol, 0.6 mL) and a catalytic amount of *p*-toluensulfonic acid were added. The mixture was stirred at reflux for 24 h using a Dean-Stark trap. After cooling, the organic phase was washed with a saturated solution of sodium hydrogen carbonate (3 x 20 mL), dried (Na_2SO_4) and evaporated under reduced pressure. The residue was purified by Biotage chromatography using cyclohexane/ethyl acetate (98/2) as eluent. For compounds **9c,e** see supporting information.

General procedure for 2-(1,3-dioxolan-2-yl)benzaldehydes (10c,e).[52] For compounds **10c, e** see supporting information.

General procedure for substituted benzene-1,2-dicarbaldehydes (3c,e). To a solution of suitable derivatives **10c,e** (1.5 mmol) in acetone (17 mL) a catalytic amount of *p*-toluensulfonic acid was added. The solution was stirred for 15 min at room temperature. The solvent was evaporated under reduced pressure and the residue was dissolved in dichloromethane (20 mL) and washed with saturated solution of sodium carbonate (3 x 20 mL). The organic phase was dried (Na_2SO_4), evaporated under *vacuo* and the residue was purified by chromatography using dichloromethane as eluent. For compounds **3c, e** see supporting information.

General procedure for 2-(2'-aminophenyl)-2*H*-isoindole -1-carbonitriles (5a,f,h-n,o-q). To a solution of sodium hydrogen sulfite (0.015mol, 1.56 g) in water (38 mL), an appropriate 1,2-

dicarbaldehyde **3a-e** (0.015 mol) was added. The mixture was stirred until the solid was dissolved, and then the suitable diamine **4c,e,g** (0.015 mol) was added. The reaction was stirred at room temperature for 3-24 h or heated at 40-60 °C for 30 min. Then potassium cyanide (0.052 mol, 3.39 g) in water (8.0 mL) was added, and the mixture was stirred at room temperature for 24 h or heat at 40-60 °C for 90 min. The solid formed upon cooling was filtered and purified by chromatography using dichloromethane (derivatives **5a,f, i,m,o-q**), dichloromethane/ethyl acetate (8/2) (derivative **5n**) or cyclohexane/ethyl acetate (8/2) (derivatives **5h,j,k,l**) as eluent.

2-(2-Amino-5-methoxyphenyl)-2*H*-isoindole-1-carbonitrile (5a). Conditions: 30 min 60 °C before and 90 min 60 °C after KCN addition. Brown solid; yield: 47%; m.p,: 105-106 °C; IR (cm^{-1}) 3452, 3365, 2200; ^{1}H NMR (200 MHz, $CDCl_3$) δ: 3.38 (bs, 2H, NH_2), 3.77 (s, 3H, OCH_3), 6.82-6.96 (m, 3H, H-3', H-4', H-6'), 7.17(t, 1H, *J* = 8.0 Hz, H-5), 7.27 (t, 1H, *J* = 8.0 Hz, H-6), 7.47 (s, 1H, H-3), 7.67-7.74 (m, 2H, H-4, H-7); ^{13}C NMR (50 MHz, $CDCl_3$) δ: 55.9 (q), 95.0 (s), 112.7 (d), 113.8(s), 117.8 (d), 118.2 (d), 118.3(d), 120.4 (d), 120.9 (d), 123.3 (d), 124.4 (s), 124.5 (s), 126.0 (d), 131.8 (s), 135.7 (s), 152.5 (s). Anal. Calcd for $C_{16}H_{13}N_3O$: C, 72.99; H, 4.98; N, 15.96. Found: C, 72.73; H, 5.36; N, 15.67.

2-(2-Amino-3,6-dimethoxyphenyl-2*H*-isoindole-1-carbonitrile (5f). Conditions: 24 h rt before and after KCN addition. Brown solid; yield: 50%; m.p. : 162-163 °C; IR (cm^{-1}) 3486, 3389, 2202; ^{1}H NMR (200 MHz, $CDCl_3$) δ: 3.64 (s, 2H, NH_2), 3.61 (s, 3H, OCH_3), 3.81 (s, 3H, OCH_3), 6.35 (d, 1H, *J* = 8.0 Hz, H-5'), 6.95 (d, 1H, *J* = 8.0 Hz, H-4'), 7.09-7.17 (m, 1H, H-6), 7.24-7.32 (m, 1H, H-5), 7.64 (d, 1H, *J* = 8.0 Hz, H-4), 7.73-7.78 (m, 2H, H-3, H-7) ; ^{1}H NMR (50 MHz, $CDCl_3$) δ: 55.6 (q), 56.0 (q), 94.7 (s), 94.2 (d), 98.3 (d), 103.4 (d), 104.6 (d), 114.4 (d), 115.8 (s),

119.7 (s), 116.8 (s), 128.2 (d), 128.9 (d), 142.5 (s), 148.8 (s), 152.1 (s), 161.4 (s). Anal. Calcd for $C_{17}H_{15}N_3O_2$: C, 69.61; H, 5.15; N, 14.33. Found: C, 69.40; H, 5.03; N, 13.91.

2-(2-Amino-4-methoxyphenyl)-5,6-dimethyl-2*H*-isoindole-1-carbonitrile (5h). Conditions: 30 min 40 °C before and 90 min 40 °C after KCN addition. Brown solid; yield: 85%; m.p.: 179 -180 °C; IR (cm^{-1}) 3487, 3392, 2195; ^1H NMR (200 MHz, $CDCl_3$) δ: 2.34 (s, 3H, CH_3), 2.38 (s, 3H, CH_3), 3.63 (bs, 2H, NH_2), 3.79 (s, 3H, OCH_3), 6.35-6.42 (m, 2H, H-5', H-3'), 7.11 (d, 1H, *J* = 8.5 Hz, H-6'), 7.25 (s, 1H, H-3), 7.40-7.45 (m, 2H, H-4, H-7); ^{13}C NMR (50 MHz, $CDCl_3$) δ: 20.5 (q), 20.9 (q), 55.5 (q), 94.3 (s), 101.4 (d), 104.4 (d), 114.5 (s), 117.2 (d), 117.7 (s), 119.5 (d), 119.9 (d), 124.0 (s), 128.8 (d), 131.4 (s), 133.2 (s), 136.4 (s), 143.5 (s), 161.4 (s). Anal. Calcd for $C_{18}H_{17}N_3O$: C, 74.20; H, 5.88; N, 14.42. Found: C, 74.12; H, 5.70; N, 14.16.

2-(2-Amino-4-methoxyphenyl)-5,6-dimethoxy-2*H*-isoindole-1-carbonitrile (5i). Conditions: 30 min 40 °C before and 90 min 40 °C after KCN addition. Brown solid; yield: 90%; m.p.: 223-224 °C; IR (cm^{-1}) 3469, 3371, 2200; ^1H NMR (200 MHz, $CDCl_3$) δ: 3.64 (bs, 2H, NH_2), 3.82 (s, 3H, OCH_3), 3.93 (s, 3H, OCH_3), 3.98 (s, 3H, OCH_3), 6.39-6.44 (m, 2H, H-3', H-5'), 6.85-6.94 (m, 2H, H-4, H-7), 7.11-7.26 (m, 2H, H-3, H-6'); ^{13}C NMR (50 MHz, $CDCl_3$) δ: 55.5 (q), 55.9 (q), 56.1 (q), 94.7 (s), 96.2 (d), 98.3 (d), 101.4 (d), 104.4 (d), 114.5 (s), 117.8 (s), 119.7 (s), 119.8 (s), 128.2 (d), 128.9 (d), 143.5 (s), 148.8 (s), 151.1 (s), 161.3 (s). Anal. Calcd for $C_{18}H_{17}N_3O_3$: C, 66.86; H, 5.30; N, 13.00. Found: C, 67.12; H, 5.09; N, 12.76.

2-(2-Amino-4-methoxyphenyl)-7-fluoro-2*H*-isoindole-1-carbonitrile (5j). Conditions: 30 min 40 °C before and 90 min 40 °C after KCN addition. Brown solid; yield: 65%; m.p. :118-119 °C; IR (cm^{-1}) 3481, 3381, 2212;; ^1H NMR (200 MHz, $CDCl_3$) δ: 3.65 (bs, 2H, NH_2), 3.81 (s, 3H, OCH_3), 6.39-6.43 (m, 1H, H-5'), 6.47 (d, 1H, *J* = 2.0 Hz, H-3'), 6.87-6.96 (m, 1H, H-5), 7.02-7.10 (m, 1H, H-6), 7.15 (d, 1H, *J* = 8.0 Hz, H-4), 7.43-7.47 (m, 2H, H-3, H-6'); ^{13}C NMR (50

MHz, $CDCl_3$) δ: 55.5 (q), 101.6 (d), 104.8 (d), 108.7 (d, J_F = 17.0 Hz), 113.5 8s), 116.8 (d), 117.0 (d, J_F = 11.0 Hz), 121.4 (d), 122.4 (s), 123.4 (s), 123.5 (d), 127.1 (d, J_F = 6.0 Hz), 128.8 (d), 143.3 (s), 154.6 (d, J_F = 253.0 Hz), 161.7 (s). Anal. Calcd for $C_{16}H_{12}FN_3O$: C, 68.32; H, 4.30; N, 14.94. Found: C, 68.03; H, 4.09; N, 14.80.

2-(2-Amino-4-methoxyphenyl)-6-methoxy-2*H*-isoindole-1-carbonitrile (5k). Conditions: 30 min 40 °C before and 90 min 40 °C after KCN addition. Brown solid; yield: 42%; m.p.: 146-147°C; IR (cm^{-1}) 3477, 3423, 2200; ^1H NMR (200 MHz, $CDCl_3$) δ: 3.65 (bs, 2H, NH_2), 3.82 (s, 3H, OCH_3), 3.89 (s, 3H, OCH_3), 6.38-6.45 (m, 2H, H-3', H-5'), 6.85 (dd, 1H, *J* = 8.8, 2.2 Hz, H-5), 6.93 (d, 1H, *J* = 2.2 Hz, H-7), 7.14 (d, 1H, *J* = 8.8 Hz, H-4), 7.32 (s, 1H, H-3), 7.55 (dd, 1H, *J* = 9.1, 0.56 Hz, H-6'); ^{13}C (50 MHz, $CDCl_3$) δ: 55.4 (q), 55.5 (q), 99.9 (s), 95.0 (d), 101.5 (d), 104.5 (d), 114.5 (s), 117.7 (s), 118.4 (d), 120.6 (s), 121.2 (d), 122.2 (d), 128.8 (d), 133.2 (s), 143.4 (s), 158.5 (s), 161.4 (s). Anal. Calcd for $C_{17}H_{15}N_3O_2$: C, 69.61; H, 5.15; N, 14.33. Found: C, 69.35; H, 5.44; N, 14.28.

2-(2-Amino-4-methoxyphenyl)-5-methoxy-2*H*-isoindole-1-carbonitrile (5l). Conditions: 30 min 40 °C before and 90 min 40 °C after KCN addition. Brown solid; yield: 42%; m.p.: 144-145 °C; IR (cm^{-1}) 3465, 3375, 2198; ^1H NMR (200 MHz, $CDCl_3$) δ: 3.63 (bs, 2H, NH_2), 3.83 (s, 3H, OCH_3), 3.85 (s, 3H, OCH_3), 6.38-6.45 (m, 2H, H-3', H-5'), 6.90 (d, 1H, *J* = 2.1 Hz, H-4), 7.00 (dd, 1H, *J* = 9.1, 2.1 Hz, H-6), 7.26-7.28 (m, 2H, H-3, H-6'), 7.61-768 (m, 1H, H-7); ^{13}C NMR (50 MHz, $CDCl_3$) δ: 55.3 (q), 55.5 (q), 97.2 (d), 101.4 (d), 104.5 (d), 113.9 (s), 117.7 (s), 118.1 (s), 119.5 (d), 119.6 (d), 120.8 (d), 124.9 (s), 127.9 (s), 128.8 (d), 143.3 (s), 156.2 (s), 161.4 (s). Anal. Calcd for $C_{17}H_{15}N_3O_2$: C, 69.61; H, 5.15; N, 14.33. Found: C, 69.33; H, 5.09; N, 14.09.

2-(2-Amino-4-hydroxyphenyl)-5,6-dimethyl-2*H*-isoindole-1-carbonitrile (5m). Conditions: 3 h rt before and 90 min 40 °C 24 h rt after KCN addition. Brown solid; yield: 40%; m.p.: 197-198 °C; IR (cm^{-1}) 3487, 3392, 3328, 2196; ^1H NMR (200 MHz, DMSO-d_6) δ: 2.30 (s, 3H,

CH_3), 2.34 (s, 3H, CH_3), 4.92 (bs, 2H, NH_2), 6.12 (dd, 1H, *J* = 8.5, 2.5 Hz, H-5'), 6.32 (d, 1H, *J* = 2.5 Hz, H-3'), 6.94 (d, 1H, *J* = 8.5 Hz, H-6'), 7.39 (s, 1H, H-4), 7.47 (s, 1H, H-7), 7.57 (s, 1H, H-3), 9.50 (s, 1H, OH);^{13}C NMR (50 MHz, DMSO-d_6) δ: 20.0 (q), 20.3 (q), 93.2 (s), 101.6 (d), 103.8 (d), 114.6 (s), 115.4 (s), 116.3 (d), 120.0 (d), 121.5 (d), 123.4 (s), 128.6 (d), 130.7 (s), 131.8 (s), 135.5 (s), 145.1 (s), 159.0 (s). Anal. Calcd for $C_{17}H_{15}N_3O$: C, 73.63; H, 5.45; N, 15.15. Found: C, 73.42; H, 5.74; N, 15.33.

2-(2-Amino-4-hydroxyphenyl)-5,6-dimethoxy-2*H*-isoindole-1-carbonitrile (5n). Conditions: 24 h rt before and after KCN addition. Brown solid; yield: 60%**;** m.p.: 202-203 °C; IR (cm^{-1}) 3467, 3384, 3326, 2186; ^{1}H NMR (200 MHz, DMSO-d_6) δ: 3.79 (s, 3H, OCH_3), 3.86 (s, 3H, OCH_3), 4.92 (bs, 2H, NH_2), 6.09 (dd, 1H, *J* = 8.5, 2.5 Hz, H-5'), 6.30 (d, 1H, *J* = 2.5 Hz, H-3'), 6.90 (s, 1H, H-4), 6.92 (d, 1H, *J* = 8.5 Hz, H-6'), 7.05 (s, 1H, H-7), 7.48 (s, 1H, H-3), 9.46 (s, 1H, OH); ^{13}C NMR (50 MHz, DMSO-d_6) δ: 55.3 (q), 55.5 (q), 93.6 (s), 95.8 (d), 99.3 (d), 101.6 (d), 103.7 (d), 114.8 (s), 115.5 (s), 119.1 (s), 121.4 (d), 127.3 (s), 128.6 (d), 145.2 (s), 148.0 (s), 150.7 (s), 158.9 (s). Anal. Calcd for $C_{17}H_{15}N_3O_3$: C, 66.01; H, 4.89; N, 13.58. Found: C, 65.76; H, 4.95; N, 13.77.

2-(2-Amino-4-hydroxyphenyl)-7-fluoro-2*H*-isoindole-1-carbonitrile (5o). Conditions: 3 h rt before and 24 h rt after KCN addition. Brown solid; yield: 60%; m.p.: 204-205 °C; IR (cm^{-1}) 3482, 3376, 3234, 2217; ^{1}H NMR (200 MHz, DMSO-d_6) δ: 5.09 (bs, 2H, NH_2), 6.10 (dd, 1H, *J* = 8.5, 2.5 Hz, H-5'), 6.30 (d, 1H, *J* = 2.5 Hz, H-3'), 6.98 (d, 1H, *J* = 8.5 Hz, H-6'), 7.02-7.14 (m, 2H, H-5, H-4), 7.58 (dt, 1H, J_F = 2.5 Hz, *J* = 6.7 Hz, H-6), 7.87 (1H, d, J_F = 2.5 Hz, H-3), 9.52 (s, 1H, OH); ^{13}C NMR (50 MHz, DMSO-d_6) δ: 92.1 (d, J_F = 2.8 Hz), 101.6 (d), 103.7 (d), 108.1 (dd, J_F = 16.7 Hz), 114.0 (s), 114.7 (s), 117.9 (dd, J_F = 4.3 Hz), 121.1 (d, J_F = 18 Hz), 122.4 (dd, J_F = 6.4 Hz), 123.7 (d), 126.7 (d, J_F = 6.7 Hz), 128.7 (d), 145.3 (s), 153.7 (d, J_F = 248 Hz), 159.3 (s). Anal. Calcd for $C_{15}H_{10}FN_3O$: C, 67.41; H, 3.77; N, 15.72. Found: C, 67.70; H, 4.06; N, 15.96.

2-(2-Amino-4-hydroxyphenyl)-6-methoxy-2*H*-isoindole-1-carbonitrile (5p). Conditions: 3 h rt before and 24 h rt after KCN addition. Brown solid; yield: 43%; m.p.: 211-212 °C; IR (cm^{-1}); 3430, 3345, 3303, 2200 ^{1}H NMR (200 MHz, DMSO-d_6) δ: 3.79 (s, 3H, OCH_3), 4.92 (bs, 2H, NH_2), 6.10 (dd, 1H, *J* = 8.5, 2.5 Hz, H-5'), 6.30 (d, 1H, *J* = 2.5 Hz, H-3'), 6.94 (d, 1H, *J* = 8.5 Hz, H-6'), 6.96 (dd, 1H, *J* = 9.1, 2.2, Hz, H-5), 7.07 (d, 1H, *J* = 2.2 Hz, H-7), 7.54 (d, 1H, *J* = 9.1 Hz, H-4), 7.58 (s, 1H, H-3), 9.49 (s, 1H, OH); ^{13}C NMR (50 MHz, DMSO-d_6) δ: 55.0 (q), 94.5 (s), 98.2 (d), 101.6 (d), 103.7 (d), 114.2 (s), 115.3 (s), 118.9 (d), 120.0 (d), 121.3 (d), 124.3 (s), 127.1 (s), 128.6 (d), 145.2 (s), 155.1 (s), 159.0 (s). Anal. Calcd for $C_{16}H_{13}N_3O_2$: C, 68.81; H, 4.69; N, 15.05. Found: C, 69.12; H, 5.03; N, 15.32.

2-(2-Amino-4-hydroxyphenyl)-5-methoxy-2*H*-isoindole-1-carbonitrile (5q). Conditions: 3 h rt before and 24 h rt after KCN addition. Brown solid; yield: 40%; m.p: 194-196°C; IR (cm^{-1}) 3475, 3372, 3266, 2198; ^{1}H NMR (200 MHz, DMSO-d_6) δ: 3.84 (s, 3H, OCH_3), 4.97 (bs, 2H, NH_2), 6.09 (dd, 1H, *J* = 8.5, 2.5 Hz, H-5'), 6.29 (d, 1H, *J* = 2.5 Hz, H-3'), 6.79 (dd, 1H, *J* = 9.0 2.2 Hz, H-5), 6.89 (d, 1H, *J* = 2.2 Hz, H-7), 6.93 (d, 1H, *J* = 8.5 Hz, H6'), 7.64 (d, 1H, *J* = 9.0 Hz, H-4), 7.66 (s, 1H, H-3), 9.47 (s, 1H, OH); ^{13}C NMR (50 MHz, DMSO-d_6) δ: 55.2 (q), 93.8 (s), 94.7 (d), 101.6 (d), 103.7 (d), 114.7 (s), 115.3 (s), 116.9 (d), 120.0 (s), 121.3 (d), 123.0 (d), 128.6 (d), 132.3 (s), 145.2 (s), 157.9 (s), 159.0 (s). Anal. Calcd for $C_{16}H_{13}N_3O_2$: C, 68.81; H, 4.69; N, 15.05. Found: C, 69.10; H, 4.77; N, 15.33.

General procedure for isoindolo[2,1-*a*]quinoxalin-6(5*H*)-iminium acetates (1a-i,m-p).

A solution of suitable 2-(2'-aminophenyl)-2*H*-isoindole-1-carbonitrile **5a-i,m-p** (3 mmol) in acetic acid (10 mL) was stirred under reflux for 30 min. The precipitate formed after cooling was filtered, collected and crystallized in ethanol to gave the desired compounds **1a-i,m-p**.

2-Methoxyisoindolo[2,1-*a*]quinoxalin-6(5*H*)-iminium acetate (1a). Yellow solid; yield: 99%; m.p.: 182 -182 °C; IR (cm^{-1}) 3500, 3347; ^1H NMR (200 MHz, DMSO-d_6) δ: 1.91 (s, 3H, CH_3), 3.94 (s, 3H, OCH_3), 6.70 (bs, 2H, NH_2), 7.12 (dd, 1H, J = 8.9, 2.7 Hz, H-3), 7.28-7.33 (m, 2H, H-8, H-9), 7.53 (d, 1H, J = 8.9 Hz, H-4), 7.86-7.90 (m, 1H, H-7), 7.91 (d, 1H, J = 2.7 Hz, H-1), 8.41-8.45 (m, 1H, H-10), 8.98 (s, 1H, H-11), 11.93 (s, 1H, NH); ^{13}C NMR (50 MHz, DMSO-d_6) δ: 21.0 (q), 55.9 (q), 99.2 (d), 108.1 (d), 115.6 (d). 116.7 (s), 119.6 (d), 120.4 (d), 122.6 (d), 123.8 (d), 125.8 (s), 126.3 (d), 131.7 (s), 149.8 (s), 155.2 (s), 155.7 (s), 159.7 (s), 172.0 (s). Anal. Calcd for $C_{18}H_{17}N_3O_3$: C, 66.86; H, 5.30; N, 13.00. Found: C, 66.66; H, 5.25; N, 12.73.

Isoindolo[2,1-*a*]quinoxalin-6(5*H*)-iminium acetate (1b). Yellow solid; yield: 99%;m.p: 249-250 °C; IR (cm^{-1}) 3501, 3367; ^1H NMR (200 MHz, DMSO-d_6) δ:1.91 (s, 1H, CH_3), 6.87 (bs, 2H, NH_2), 7.28-7.36 (m, 3H, H-1, H-4, H-2), 7.46 (dt, 1H, J = 7.0, 2.0, Hz, H-3), 7.58 (dd, 1H, J = 8.1, 1.2, Hz, H-7), 7.86-7.90 (m, 1H, H-9), 8.38 (d, 1H, J = 8.1 Hz, H-8), 8.42-8.47 (m, 1H, H-10), 8.93 (s, 1H, H-11), 11.90 (s, 1H, NH); ^3C NMR (50 MHz, DMSO-d_6) δ:21.1 (q), 117.7 (d), 108.0 (s), 115.64 (d), 119.4 (d), 119.5 (s), 120.2 (d), 122.1 (d), 122.7 (d), 123.1 (d), 123.6 (s), 125.5 (d), 125.7 (s), 126.9 (d), 138.4 (s), 151.4 (s), 172.0 (s). Anal. Calcd for $C_{17}H_{15}N_3O_2$: C, 69.61; H, 5.15; N, 14.33. Found: C, 69.38; H, 5.03; N, 14.07.

4-Methylisoindolo[2,1-*a*]quinoxalin-6(5*H*)-iminium acetate (1c). Yellow solid; yield: 95%; m.p: 210-212 °C; IR (cm^{-1}) 3309, 3320; ^1H NMR (200 MHz, DMSO-d_6) δ:1.91 (s, 3H, CH_3), 2.60 (s, 3H, CH_3), 6.81 (bs, 2H, NH_2), 7.17-7.37 (m, 4H, H-1, H-2, H-3, H-7), 7.85-7.89 (m, 1H, H-9), 8.22 (d, 1H, J = 7.7 Hz, H-8), 8.43-8.48 (m, 1H, H-10), 8.89 (s, 1H, H-11), 11.96 (bs, 1H, NH); ^{13}C NMR (50 MHz, DMSO-d_6) δ:21.02 (q), 18.23 (q), 99.49 (d), 107.97 (s), 113.39 (d), 119.36 (d), 119.51 (s), 120.23 (d), 121.58 (d), 122.15 (s), 122.54 (d), 123.05 (d), 123.28 (s),

123.83 (s), 127.59 (d), 133.39 (s), 150.37 (s), 171.97 (s). Anal. Calcd for $C_{18}H_{17}N_3O_2$: C, 70.34; H, 5.58; N, 13.67. Found: C, 70.14; H, 5.51; N, 13.89.

3-Methoxyisoindolo[2,1-*a*]quinoxalin-6(5*H*)-iminium acetate (1d). Yellow solid, yield: 99%; m.p.: 243-244 °C; IR (cm^{-1}) 3303, 3500; H NMR (200 MHz, DMSO-d_6) δ: 1.92 (s, 3H, CH_3), 3.86 (s, 3H, OCH_3), 6.83 (bs, 2H, NH_2), 6.93 (dd, 1H, J = 8.9, 2.8 Hz, H-2), 7.04 (d, 1H, J = 2.8 Hz, H-4), 7.28-7.33 (m, 2H, H-1, H-8), 7.82-7.87 (m, 1H, H-9), 8.29 (d, 1H, J = 9.0 Hz, H-7), 8.40-8.44 (m, 1H, H-10), 8.82 (s, 1H, H-11), 12.04 (bs, 1H, NH); ^{13}C NMR (50 MHz, DMSO-d_6) δ: 21.1 (q), 55.3 (q), 107.5 (d), 107.5 (d), 110.4 (d), 116.6 (d), 117.9 (s), 118.6 (s), 119.2 (d), 119.5 (s), 120.0 (d), 122.4 (d), 122.8 (d), 125.7 (s), 129.9 (s), 151.7 (s), 158.2 (s), 172.0 (s). Anal. Calcd for $C_{18}H_{17}N_3O_3$: C, 66.86; H, 5.30; N, 13.00. Found: C, 66.63; H, 5.36; N, 12.71.

2,3-Dimethylisoindolo[2,1-*a*]quinoxalin-6(5*H*)-iminium acetate (1e). Brown solid; yield: 90%; m.p.: 238-239 °C; IR (cm^{-1}) 3362, 3401; ^{1}H NMR (200 MHz, DMSO-d_6) δ: 1.92 (s, 3H, CH_3), 2.34 (s, 3H, CH_3), 2.40 (s, 3H, CH_3), 6.71 (bs, 2H, NH_2), 7.25-7.30 (m, 2H, H-8, H-9), 7.37 (s, 1H, H-4), 7.83-7.88 (m, 1H, H-7), 8.11 (s, 1H, H-1), 8.39-8.43 (m, 1H, H-10), 8.83 (s, 1H, H-11) 11.95 (s, 1H, NH); ^{13}C NMR (50 MHz, DMSO-d_6) δ: 21.1 (q), 55.3 (qx2), 106.9 (d), 108.1 (s), 115.8 (d), 119.3 (d), 119.4 (s), 120.1 (d), 121.5 (s), 122.3 (d), 122.9 (d), 125.7 (s), 125.7 (d), 131.0 (s), 135.5 (s), 136.4 (s), 151.0 (s), 172.0 (s). Anal. Calcd for $C_{19}H_{19}N_3O_2$: C, 71.01; H, 5.96; N, 13.08. Found: C, 70.75; H, 5.75; N, 12.82.

1,4-Dimethoxyisoindolo[2,1-*a*]quinoxalin-6(5*H*)-iminium acetate (1f). Yellow solid; yield: 90%; m.p.: 272-273 °C; IR (cm^{-1}) 3352, 3339; ^{1}H NMR (200 MHz, DMSO-d_6) δ: 1.92 (s, 3H, CH_3), 3.87 (s, 3H, OCH_3), 4.06 (s, 3H, OCH_3), 6.87-7.02 (m, 4H, NH_2, H-2, H-3), 7.25-7.35 (m, 2H, H-8, H-9), 7.90-7.95 (m, 1H, H-7), 8.41-8.45 (m, 1H, H-10), 9.38 (s, 1H, H-11). 11.01 (s,

1H, NH); ^{13}C NMR (50 MHz, DMSO-d_6) δ: 21.0 (q), 55.8 (q), 56.4 (q), 103.3 (d), 107.5 (d), 108.3 (s), 113.9 (d), 114.9 (s), 118.2 (s), 119.7 (d), 119.7 (d), 122.7 (d), 122.9 (d), 125.2 (s), 130.9 (s), 144.6 (s), 147.4 (s), 150.7 (s), 172.9 (s). Anal. Calcd for $C_{19}H_{19}N_3O_4$: C, 64.58; H, 5.42; N, 11.89. Found: C, 64.32; H, 5.28; N, 11.84.

2,3-Dichloroisoindolo[2,1-*a*]quinoxalin-6(5*H*)-iminium acetate (1g). Yellow solid; yield: 99%; m.p.: 242-243 °C; IR (cm^{-1}) 3557, 3398; ^{1}H NMR (200 MHz, DMSO-d_6) δ: 1.92 (s, 3H, CH_3), 7.14 (bs, 2H, NH_2), 7.30-7.35 (m, 2H, H-8, H-9), 7.69 (s, 1H, H-4), 7.83-7.89 (m, 1H, H-10), 8.45 (s, 1H, H-7), 8.77 (s, 1H, H-1), 9.03 (s, 1H, H-11), 11.07 (s, 1H, NH); ^{13}C NMR (50 MHz, DMSO-d_6) δ: 21.02 (q), 99.34 (s), 107.7 (d), 109.2 (s), 117.6 (s), 119.7 (s), 119.9 (d), 120.1 (s), 123.1 (d), 123.3 (s), 123.5 (d), 123.5 (s), 125.9 (s), 128.9 (d), 138.8 (d), 152.3 (d) 172.0 (s). Anal. Calcd for $C_{17}H_{13}Cl_2N_3O_2$: C, 56.37; H, 3.62; N, 11.60. Found: C, 56.14; H, 3.33; N, 11.86.

3-Methoxy-8,9-dimethylisoindolo[2,1-a]quinoxalin-6(5*H*)-iminium acetate (1h). Yellow solid; yield: 95%; m.p: 243-244 °C; IR (cm^{-1}) 3399, 3398; ^{1}H NMR (200 MHz, DMSO-d_6) δ: 1.91 (s, 3H, CH_3), 2.38 (s, 3H, CH_3), 2.42 (s, 3H, CH_3), 3.84 (s, 3H, OCH_3), 6.74 (bs, 2H, NH_2), 6.89 (dd, 1H, J = 8.9, 2.8 Hz, H-2), 7.00 (d, 1H, J = 2.8 Hz, H-4), 7.58 (s, 1H, H-7), 8.19-8.23 (m, 2H, H-1, H-10), 8.64 (s, 1H H-11) 11.06 (s, 1H, NH); ^{13}C NMR (50 MHz, DMSO-d_6) δ: 20.2 (q), 20.38 (q), 21.1 (q), 55.2 (q), 106.3 (d), 106.8 (s), 107.5 (d), 110.1 (d), 116.4 (d), 118.1 (d), 119.0 (d), 119.1 (s), 125.2 (s), 132.1 (s), 132.3 (s), 139.8 (s), 151.6 (s), 158.0 (2xs), 172.0 (s). Anal. Calcd for $C_{20}H_{21}N_3O_3$: C, 68.36; H, 6.02; N, 11.96. Found: C, 68.13; H, 6.23; N, 11.76.

3,8,9-Trimethoxyisoindolo[2,1-*a*]quinoxalin-6(5*H*)-iminium acetate (1i). Yellow solid; yield 99%; m.p: 357-358 °C; IR (cm^{-1}) 3439, 3366; ^{1}H NMR (200 MHz, DMSO-d_6) δ: 1.91 (s, 1H, CH_3), 3.84 (s, 3H, OCH_3), 3.85 (s, 3H, OCH_3), 3.95 (s, 3H, OCH_3), 6.77 (bs, 2H, NH_2), 6.87 (dd,

1H, *J* = 8.9, 2.8 Hz, H-2), 6.97 (d, 1H, *J* = 2.8, H-4), 7.14 (s, 1H, H-7), 7.63(s, 1H, H-10), 8.13 (d, 1H, *J* = 8.9 Hz, H-1), 8.55 (s, 1H, H-11) 11.96 (s, 1H, NH); ^{13}C NMR (50 MHz, DMSO-d_6) δ: 21.1 (q), 55.2 (q), 55.8 (q), 56.0 (q), 97.7 (d), 99.2 (d), 106.5 (d), 107.4 (s), 107.5 (d), 109.8 (d), 115.0 (s), 115.9 (d), 118.4 (s), 121.4 (s), 139.3 (s), 148.3 (s), 148.5 (s), 151.5 (s), 157.6 (s), 172.1 (s). Anal. Calcd for $C_{20}H_{21}N_3O_5$: C, 62.65; H, 5.52; N, 10.96. Found: C, 62.90; H, 5.46; N, 10.75.

3-Hydroxy-8,9-dimethylisoindolo[2,1-*a*]quinoxalin-6(5*H*)-iminium acetate (1m). Yellow solid; yield: 55%; m.p.: 289.1-289.4°C; IR (cm^{-1}) 3390, 3284, 3264; ^{1}H NMR (200 MHz, DMSO-d_6) δ: 1.91 (s, 3H, CH_3), 2.38 (s, 3H, CH_3), 2.42 (s, 3H, CH_3), 6.77 (bs, 2H, NH_2), 6.78 (dd, 1H, *J* = 8.8, 2.6 Hz, H-2), 6.91 (d, 1H, *J* = 2.6 Hz, H-4), 7.59 (s,1H, H-7), 8.10 (d, 1H, *J* = 8.8 Hz, H-1), 8.14 (s, 1H, H-10), 8.57 (s, 1H, H-11), 9.08 (bs, 1H, OH), 9.59 (bs, 1H, NH); ^{13}C NMR (50 MHz, DMSO-d_6) δ: 20.2 (q), 20.4 (q), 21.1 (q), 106.3 (d), 106.7 (s), 109.4 (d), 110.9 (d), 116.2 (d), 117.1 (s), 118.1 (d), 119.0 (d), 119.1 (s), 125.1 (s), 132.0 (s), 132.1 (s), 139.6 (s), 151.4 (s), 156.1 (s), 172.1 (s). Anal. Calcd for $C_{19}H_{19}N_3O_3$: C, 67.64.; H, 5.68; N, 12.45. Found: C, 68.42; H, 5.55; N, 12.44.

3-Hydroxy-8,9-dimethoxyisoindolo[2,1-*a*]quinoxalin-6(5*H*)-iminium acetate (1n). Yellow solid; yield 99%; m.p: 243-244 °C; IR (cm^{-1}) 3677, 3557, 3348; ^{1}H NMR (200 MHz, DMSO-d_6) δ: 1.91 (s, 3H, CH_3), 3.85 (s, 3H, OCH_3), 3.92 (s, 3H, OCH_3), 6.68 (m, 3H, NH_2, H-2), 6.86 (d, 1H, *J* = 2.4 Hz, H-4), 7.13 (s, 1H, H-7), 7.61 (s, 1H, H-10), 8.02 (d, 1H, *J* = 8.9 Hz, H-1), 8.49 (s, 1H, H-11), 9.43-9.66 (m, 2H, OH, NH); ^{13}C NMR (50 MHz, DMSO-d_6) δ: 21.1 (q), 55.2 (q), 55.8 (q), 97.7 (d), 99.5 (d), 106.0 (d), 107.3 (s), 109.4 (d), 110.7 (d), 114.9 (d), 115.7 (s), 117.5 (s), 118.7 (s), 121.2 (s), 139.3 (s), 148.2 (s), 151.4 (s), 155.7 (s), 172.0 (s). Anal. Calcd for $C_{19}H_{19}N_3O_5$: C, 61.78; H, 5.18; N, 11.38. Found: C, 61.52; H, 5.13; N, 11.64.

10-Fluoro-3-hydroxyisoindolo[2,1-*a*]quinoxalin-6(5*H*)-iminium acetate (1o).Yellow solid; yield: 86 %; m.p: 340 °C; IR (cm^{-1}) 3387, 3264, 3252; ^{1}H NMR (200 MHz, DMSO-d_6) δ:1.91 (s, 3H, CH_3), 6.78 (bs, 2H, NH_2), 6.81 (dd, 1H, J = 8.9, 2.4 Hz, H-2), 6.93 (d, 1H, J = 2.4 Hz, H-4), 7.03-7.29 (m, 3H, H-11, H-8, H-9), 7.69 (d, 1H, J = 8.2 Hz, H-7), 8.20 (d, 1H, J = 8.9 Hz, H-1) 8.92 (bs, 1H, OH), 10.60 (bs, 1H, NH); ^{13}C NMR (50 MHz, DMSO-d_6) δ:21.04 (s), 99.5 (s), 105.8 (d, J_F = 4.1 Hz), 106.5 (d, J_F = 22.9 Hz), 108.4 (d), 108.8 (d, J_F =16.8 Hz), 109.5 (d), 111.7 (d), 116.3 (d, J_F = 3.6 Hz), 116.4 (s), 116.8 (d), 123.1 (d, J_F = 8.1 Hz), 128.6 (d, J_F = 7.4 Hz), 139.9 (s), 150.2 (s), 154.5 (d, J_F=246.4 Hz), 172.02 (s).Anal. Calcd for $C_{17}H_{14}FN_3O_3$: C, 62.38; H, 4.31; N, 12.84. Found: C, 62.59; H, 3.96; N, 12.57.

3-Hydroxy-9-methoxyisoindolo[2,1-*a*]quinoxalin-6(5*H*)-iminium acetate (1p). Yellow solid; yield: 85%; m.p: 279 -280 °C, IR (cm^{-1}) 3306, 3280, 3244; ^{1}H NMR (200 MHz, DMSO-d_6) δ:1.91 (s, 3H, CH_3), 3.92 (s, 3H, OCH_3), 6.72 (dd, 1H, J = 8.8, 2.6 Hz, H-2), 6.87 (bs, 2H, NH_2), 6.88 (d,1H, J = 2.6 Hz, H-4), 6.93 (dd, 1H, J = 9.1, 1.9 Hz, H-8), 7.63 (d, 1H, J = 1.9 Hz, H-10), 7.73 (d, 1H, J = 9.1 Hz, H-7), 8.07 (d, 1H, J = 8.8 Hz, H-1), 8.65 (s, 1H, H-11), 9.05 (bs, 1H, OH), 9.96 (bs, 1H, NH); (50 MHz, DMSO-d_6) δ: 21.1 (q), 55.5 (q), 98.2 (d), 99.5 (s), 107.2 (s), 107.4 (d), 109.3 (d), 109.6 (s),110.8 (d), 111.4 (s), 117.2 (d), 120.3 (d), 121.7 (d), 139.4 (s), 151.7 (s), 155.9 (s), 156.2 (s), 172.1 (s). Anal. Calcd for $C_{18}H_{17}N_3O_4$: C, 63.71; H, 5.05; N, 12.38. Found: C, 63.40; H, 4.74; N, 12.59.

General procedure for isoindolo[2,1-*a*]quinoxalin-6(5*H*)-imines (2a,f,h-q). A solution of suitable 2-(2'-aminophenyl)-2*H*-isoindole -1-carbonitrile **5a-q** (3 mmol) in acetic acid (10 mL) was stirred under reflux for 30 min. The mixture reaction was neutralized with saturated aqueous sodium hydrogen carbonate solution at -5 °C and then extracted with ethyl acetate. The

organic phase was dried (Na_2SO_4) and evaporated under reduced pressure. The precipitate formed was crystallized in ethanol to gave the desired compounds **2a,f,h-q.**

2-Methoxyisoindolo[2,1-*a*]quinoxalin-6(5*H*)-imine (2a). Yellow solid; yield: 90%; m.p.: 291 °C; IR (cm^{-1}) 3499, 3360; ^1H NMR (200 MHz, DMSO-d_6) δ: 3.95 (s, 3H, OCH_3), 7.27 (dd, 1H, *J* = 9.0, 2.5 Hz, H-3), 7.45-7.59 (m, 2H, H-1, H-8), 7.66 (d, 1H, *J* = 9.0 Hz, H-4), 8.03-8.10 (m, 2H, H-7, H-9), 8.48 (s, 1H, NH), 8.59 (d, 1H, *J* = 8.6 Hz, H-10), 9.46 (s, 1H, H-11), 13.52 (s, 1H, NH); Anal. Calcd for $C_{16}H_{13}N_3O$: C, 72.99; H, 4.98; N, 15.96. Found: C, 72.73; H, 5.19; N, 16.23.

1,4-Dimethoxyisoindolo[2,1-a]quinoxalin-6(5*H*)-imine (2f). Yellow solid; yield: 90%; m.p: 286-287 °C, IR (cm^{-1}) 3195, 3182; ^1H NMR (200 MHz, DMSO-d_6) δ: 4.00 (s, 3H, OCH_3), 4.10 (s, 3H, OCH_3), 7.17 (d, 1H, *J* = 9.0 Hz, H-2), 7.29 (d, 1H, *J* = 9.0 Hz, H-3), 7.47 (t, 1H, *J* = 7.9 Hz, H-8), 7.62 (t, 1H, *J* = 7.9 Hz, H-9), 8.11 (d, 1H, *J* = 7.9 Hz, H-7), 8.55 (d, 1H, *J* = 7.9 Hz, H-10), 8.93 (bs, 1H, NH), 9.66 (s, 1H, H-11), 12.06 (bs, 1H, NH). Anal. Calcd for $C_{17}H_{15}N_3O_2$: C, 69.61; H, 5.15; N, 14.33. Found: C, 69.85; H, 5.07; N, 14.54.

3-Methoxy-8,9-dimethylisoindolo[2,1-*a*]quinoxalin-6(5*H*)-imine (2h). Yellow solid; yield: 60%; m.p.: >410°C; IR (cm^{-1}) 3392, 3311; ^1H NMR (200 MHz, DMSO-d_6) δ: 2.40 (s, 3H, CH_3), 2.45 (s, 3H, CH_3), 3.89 (s, 3H, OCH_3), 7.15 (m, 2H, H-2, NH), 7.72 (s, 1H, H-7), 8.38-8.46 (m, 3H, H-1, H-4, H-11), 9.11 (s, 1H, H-10), 13.46 (bs, 1H, NH). Anal. Calcd for $C_{18}H_{17}N_3O$: C, 74.20; H, 5.88; N, 14.42. Found: C, 73.95; H, 5.83; N, 14.67.

3,8,9-Trimethoxyisoindolo[2,1-*a*]quinoxalin-6(5*H*)-imine (2i). Yellow solid; yield: 72%**;** m.p: >410°C; IR (cm^{-1}) 3675, 3556; ^1H NMR (200 MHz, DMSO-d_6) δ: 3.89 (s, 3H, OCH_3), 3.94 (s, 3H, OCH_3), 3.99 (s, 3H, OCH_3), 7.11-7.31 (m, 3H, NH, H-2, H-4), 7.82 (s, 1H, H-7), 8.35-8.67

(m, 2H, H-11, H-10), 9.16 (d, 1H, *J* = 8.6 Hz, H-1), 13.14 (bs, 1H, NH). Anal. Calcd for $C_{18}H_{17}N_3O_3$: C, 66.86; H, 5.30; N, 13.00. Found: C, 66.62; H, 5.42; N, 13.12.

10-fluoro-4-methoxyisoindolo[2,1-*a*]quinoxalin-6(5*H*)-imine (**2j**). Yellow solid; yield: 58%**;** m.p.: 246-247°C; IR (cm^{-1}) 3430, 3368; ^1H NMR (200 MHz, DMSO-d_6) δ: 3.90 (s, 3H, OCH_3), 7.16-7.20 (2H, m, H-4, H-8), 7.38-7.46 (3H, m, NH, H-2, H-9), 7.86 (d, *J* = 7.6 Hz, H-1), .8.47 (d, *J* = 8.8 Hz, H-7), 9.40 (s, 1H, H-11), 13.62 (s, 1H, NH). Anal. Calcd for $C_{16}H_{12}FN_3O$: C, 68.32; H, 4.30; N, 14.94. Found: C, 68.09; H, 4.71; N, .15.11.

3,9-Dimethoxyisoindolo[2,1-*a*]quinoxalin-6(5*H*)-imine (2k). Yellow solid; yield: 60%;m.p.: 283-284°C; IR (cm^{-1}) 3309-3181; ^1H NMR (200 MHz, DMSO-d_6) δ: 3.89 (s, 3H, OCH_3), 3.97 (s, 3H, OCH_3), 7.09-7.14 (m, 3H, NH, H-2, H-4), 7.80 (s, 1H, H-10), 7.90 (d, 1H, *J* = 8.0 Hz, H-8), 8.34-8.50 (m, 2H, H-7, H-1), 9.17 (s, 1H, H-11), 12.84 bs, (1H, NH). Anal. Calcd for $C_{17}H_{15}N_3O_2$: C, 69.61; H, 5.15; N, 14.33. Found: C, 69.78; H, 5.42; N, 14.12.

3,8-Dimethoxyisoindolo[2,1-*a*]quinoxalin-6(5*H*)-imine (2l). Yellow solid; yield: 60%; m.p.: 298-299°C; IR (cm^{-1}) 3278, 3174; ^1H NMR (200 MHz, DMSO-d_6) δ: 3.89 (s, 3H, OCH_3), 3.95 (s, 3H, OCH_3), 7.13-7.30 (m, 4H, NH, H-2, H-4, H-10), 8.40-8.50 (m, 3H, H-1, H-7, H-9), 9.08 (s, 1H, H-11), 13.16 (bs, 1H, NH). Anal. Calcd for $C_{17}H_{15}N_3O_2$: C, 69.61; H, 5.15; N, 14.33. Found: C, 69.75; H, 5.25; N, 14.15.

6-Imino-8,9-dimethyl-5,6-dihydroisoindolo[2,1-a]quinoxalin-3-ol (2m). Yellow solid; yield: 88%; m.p.: >410°C; IR (cm^{-1}) 3374, 3280, 3189; ^1H NMR (200 MHz, DMSO-d_6) δ: 2.38 (s, 3H, CH_3), 2.43 (s, 3H, CH_3), 6.95 (dd, 1H, *J* = 9.1, 2.4 Hz, H-2), 7.07 (d, 1H, *J* = 2.4 Hz, H-4), 7.71-7.73 (m, 2H, NH, H-7), 8.30 (d, 1H, *J* = 9.1 Hz, H-1), 8.32 (s, 1H, H-10), 9.03 (s, 1H, H-11), 10.40 (bs, 1H, OH), 13.01 (bs, 1H, NH). Anal. Calcd for $C_{17}H_{15}N_3O$: C, 73.63; H, 5.45; N, 15.15. Found: C, 73.92; H, 5.38; N, 15.06.

6-Imino-8,9-dimethoxy-5,6-dihydroisoindolo[2,1-*a*]quinoxalin-3-ol (2n). Yellow solid; yield: 50%; m.p: 287-288°C, IR (cm^{-1}) 3316, 3264; ^1H NMR (200 MHz, DMSO-d_6) δ: 3.88 (s, 3H, OCH_3), 3.97 (s, 3H, OCH_3), 6.93 (dd, 1H, J = 9.0, 2.3 Hz, H-2), 7.04 (d, 1H, J = 2.3 Hz, H-4), 7.26 (s, 1H, H-7), 7.75 (s, 1H, H-10), 8.21 (d, 1H, J = 9.0 Hz, H-1), 8.44 (bs, 1H, NH), 8.90 (s, 1H, H-11), 10.39 (bs, 1H, OH), 13.04 (bs, 1H, NH). Anal. Calcd for $C_{17}H_{15}N_3O_3$: C, 66.01; H, 4.89; N, 13.58. Found: C, 65.89; H, 5.13; N, 13.49.

10-Fluoro-6-imino-5,6-dihydroisoindolo[2,1-*a*]quinoxalin-3-ol (2o). Yellow solid; yield: 87%; m.p: 362-363°C; IR (cm^{-1}) 3265, 3180; ^1H NMR (200 MHz, DMSO-d_6) δ: 7.02 (dd, 1H, J = 9.0, 2.1, Hz, H-2), 7.12 (d, 1H, J = 2.1 Hz, H-4), 7.31-7.48 (m, 3H, H-8, H-9, NH), 7.84 (d, 1H, J = 7.2 Hz, H-7), 8.10-8.38 (d, 1H, J = 9.0 Hz, H-1), 9.35 (d, 1H, J_F = 1.9 Hz, H-11), 10.69 (bs, 1H, OH), 14.11 (bs, 1H, NH) Anal. Calcd for $C_{15}H_{10}FN_3O$: C, 67.41; H, 3.77; N, 15.72. Found: C, 67.32; H, 3.51; N, 15.66.

6-Imino-9-methoxy-5,6-dihydroisoindolo[2,1-*a*]quinoxalin-3-ol (2p). Yellow solid; yield: 83%; m.p: 335 -336 °C, IR: 3306, 3280; ^1H NMR (200 MHz, DMSO-d_6) δ: 3.89 (s, 3H, OCH_3), 6.94 (dd, 1H, J = 9.0, 2.3 Hz, H-2), 7.06 (d, 1H, J = 2.3 Hz, H-4), 7.09 (dd, 1H, J = 9.1, 1.7, Hz, H-8),7.80 (d, 1H, J = 1.7 Hz, H-10), 7.89 (d, 1H, J = 9.1 Hz, H-7), 8.26 (d, 1H, J = 9.0 Hz, H-1), 8.48 (bs, 1H, NH), 9.11 (s, 1H, H11), 10.44 (bs, 1H, OH), 13.05 (bs, 1H, NH). Anal. Calcd for $C_{16}H_{13}N_3O_2$: C, 68.81; H, 4.69; N, 15.05. Found: C, 68.52; H, 4.62; N, 15.29.

6-Imino-8-methoxy-5,6-dihydroisoindolo[2,1-*a*]quinoxalin-3-ol (2q). Yellow solid; yield: 89%; m.p.: 316.4, 317.7 °C; IR (cm^{-1}) 3326-3309; ^1H NMR (200 MHz, DMSO-d_6) δ: 3.89 (s, 3H, OCH_3), 7.00 (dd, 1H, J = 9.1, 2.4, Hz, H-2), 7.10 (d, 1H, J = 2.4 Hz, H-4), 7.21 (dd, 1H, J = 9.1, 2.1 Hz, H-9), 7.32 (d, 1H, J = 2.1 Hz, H-7), 8.33 (d, 1H, J = 9.1 Hz, H-1), 8.45 (bs, 1H, NH), 8.46 (d, 1H, J = 9.1 Hz , H-10), 9.05 (bs, 1H, H-11), 10.51 (bs, 1H, OH), 13.50 (bs, 1H,

NH). Anal. Calcd for $C_{16}H_{13}N_3O_2$: C, 68.81; H, 4.69; N, 15.05. Found: C, 68.49; H, 4.60; N, 15.19.

Solubility Studies of isoindolo[2,1-*a*]quinoxalin-6-imines 1 and 2. Water solubility for compounds **1** and **2** was determined by shaking an excess solute in water at 25 °C. The suspension was sonicated for 10 min, centrifuged at 8512 x g for 7 min, filtered on 0.45 μm cellulose membrane. Compound amount in saturated solution was evaluated by HPLC analysis.

BIOLOGY

Fluorescence melting studies. Melting experiments were performed in a Roche LightCycler, using an excitation source at 488 nm and recording the fluorescence emission at 520 nm. Target DNA were the human telomeric sequence (HTS) 5'-AGGGTTAGGGTTAGGGTTAGGGT-3', a c-myc based sequence (Pu24, 5'-TGAGGGTGGGGAGGGTGGGGAAGG-3', both labeled with Dabcyl at 5' end and Fluorescin at 3' end and a double stranded DNA (5'-GGATGTGAGTGTGAGTGTGAGG-3' labeled with Dabcyl at 5' end and annealed with its complementary strand labeled with Fluorescin at 3' end). Mixtures (20 μL) contained 0.25 μM of target DNA and variable ligands concentrations in 10 mM LiOH; 50 mM KCl pH 7.4 with H_3PO_4. Samples temperature was slowly increased (0.2 °C/min) up to 90 °C and again lowered at the same rate to 30 °C. Recordings were taken during both these melting and annealing reactions to check for hysteresis.

T_m values were determined from the first derivatives of the melting profiles using the Roche LightCycler software. Each curve was repeated at least three times and errors were ± 0.4 °C.

Circular dichroism measurements. Circular dichroism spectra were recorded in 10 mM Tris-HCl, 50 mM KCl, pH 7.5 from 230 to 350 nm using a 10 mm path length cell on a Jasco J 810 spectropolarimeter equipped with a Peltier temperature controller. As DNA substrates calf thymus DNA (40 μM, bp concentration, ctDNA) and the G-quadruplex forming sequence Tel22 5'-AGGGTTAGGGTTAGGGTTAGGG-3' (4 μM, strand concentration) previously annealed in the measure buffer, were used. DNA solutions were added of selected ligands and CD spectra were acquired after 15 min and after overnight incubation to check for slow oligonucleotide conformation al modifications.

The reported spectrum of each sample represents the average of 3 scans recorded with 1-nm step resolution. Observed ellipticities were converted to mean residue ellipticity [θ] = deg x cm^2 x $dmol^{-1}$ (Mol. Ellip.).

Unwinding assay. Supercoiled pBR322 DNA (0.15 μg) was incubated in the presence or absence of increasing concentrations of tested ligands in 10 mM Tris·HCl, 50 mM KCl, pH 7.5, After 30' incubation at 37°C, the reaction mixtures were loaded on 1% agarose gel, and run in TAE (40 mM TRIS, 18 mM acetic acid, 1 mM EDTA). The reaction products were visualized by Sybr Green I staining.

Cytotoxic studies. The human osteosarcoma U2Os cells were cultured in RPMI-1640 containing 10% foetal calf serum and the cytotoxicity was assessed by growth inhibition assay after 72 h drug exposure. Cells in the logarithmic phase of growth were harvested and seeded in duplicates into 6-well plates. Twenty four hours after seeding, cells were exposed to the drug and then

counted with a Coulter counter. IC_{50} is defined as the inhibitory drug concentration causing a 50% decrease of cell growth over that of untreated control.

Topoisomerase I-dependent DNA cleavage assay. A gel purified 751-bp BamHI-EcoRI fragment of SV40 DNA was used for the cleavage assay. DNA fragments were uniquely 3'-end labelled. Cleavage reactions (20,000 cpm/sample) were performed in 20 μl of 10 mM Tris-HCl (pH 7.6), 150 mM KCl, 5 mM $MgCl_2$, 15 μg/ml BSA, 0.1 mM dithiotreitol, and the human recombinant enzyme (full length topoisomerase 1)[53] for 30 min at 37ºC. Reactions were stopped by 0.5% SDS and 0.3 mg/ml of proteinase K for 45 min at 42ºC. After precipitation, DNA was resuspended in denaturing buffer (80% formamide, 10 mM NaOH, 0.01 M EDTA and 1 mg/ml dyes) before loading on a denaturing 8% polyacrylamide gel in TBE buffer. DNA cleavage was evaluated with a PhosphoImager 425 model (Molecular Dynamics).

Tubulin Polymerization Assay. Cells were seeded in 24-mm Petri dishes and were exposed the next day to the compounds for 24 h at concentrations corresponding to the IC_{50} at 72 h. Samples were then processed for the tubulin polymerization assay.[54] To separate cytosolic and cytoskeletal-associated proteins, cells were rinsed twice in PIPES-EGTA-$MgCl_2$ (PEM) buffer (85 mmol/L PIPES, pH 6.94; 10 mmol/L EGTA; 1 mmol/L $MgCl_2$; 2 mol//L glycerol; 1 mmol/L phenylmethylsulfonyl fluoride; 0.1 mmol/L leupeptin; 1 μmol/L pepstatin; 2 μg/mL aprotinin), lysed at room temperature for 10 min with PEM buffer supplemented with 0.1% v/v Triton X-100, and rinsed in PEM buffer. The Triton X-100–soluble fractions were then diluted 3:1 with 4× SDS-PAGE sample buffer. The insoluble material that remained attached to the dish was scraped into SDS-PAGE sample buffer containing protease inhibitors. Proteins were separated by SDS-PAGE, and tubulin distribution was analyzed by immunoblotting using anti α-tubulin antibody (Sigma-Aldrich).

Cell cycle phase distribution and apoptosis analyses. For cell cycle phase distribution analysis, both adherent and floating cells were fixed in 70% EtOH and incubated at 4°C for 30 minutes in staining solution containing 50 μg/mL of propidium iodide, 50 mg/mL of RNase, and 0.05% Nonidet-P40 in PBS. Samples were analyzed with a FACS-Calibur cytofluorimeter (Becton Dickinson). At least 30000 events were read and histograms were analyzed using the CellQuest software according to the Modfit model (Becton Dickinson). For apoptosis analysis, the catalytic activity of caspase-3 was measured in the same cellular samples as the ability to cleave the specific substrate N-acetyl-Asp-Glu-Val-Asp-pNA (DEVD-pNA) by means of the APOPCYTO/caspase-3 kit (MBL), according to manufacturer's instructions. The hydrolysis of the specific substrates was monitored by spectrofluorometry with 380-nm excitation and 460-nm emission filters. Results were expressed as relative fluorescence units (rfu).

Chromatin immunoprecipitation (ChIP). Cells were fixed with formaldehyde for 15 min at room temperature, harvested by scraping, and lysed in 0.1% SDS, 50 mM TRIS-HCl, pH 8.0, 10 mM EDTA, supplemented with phenylmethanesulfonylfluoride for 15 min on ice. Chromatin was chipped away in 400–800 bp-long fragments using the Branson Digital Sonifier 450 (Branson Ultrasonics Corporation). Forty μg of chromatin fragments was diluted and precleared with a Protein A/G bead mixture (GE Healthcare) for 1 h at 4°C. Chromatin was immunoprecipitated overnight at 4°C using TERF2 (Imgenex Corporation), hPOT1 (Abcam) and Topoisomerase I (BD PharMingen, San Diego, CA) antibodies. Samples were washed and eluted with 0.1 M $NaHCO_3$/1% SDS. Cross-linked proteins were released by a 6 h incubation at 65°C in 200 mM NaCl. The eluted samples were treated with 60 μg Proteinase K (Life Technologies) for 2 h at 45°C, extracted with phenol-chloroform, ethanol precipitated and recovered in 0.2 N NaOH. Samples were then blotted onto a Hybond-N filter (GE Healthcare). Telomeric DNA was

then detected using a TTAGGG probe labeled by DECA prime II Labeling kit (Life Technologies). A probe with Alu sequence was used as negative control. Equal loading was ensured by blotting 100% and 10% total chromatin (Input). Filters were washed and autoradiographed. Images were acquired by UVP Biospectrum Imaging System (Ultra-Violet Products Ltd) and quantified by VisionWorks LS software (UVP).

Statistical Analysis. Statistical evaluation of data was done with two-tailed Student's *t* test. *P*s <0.05 were considered statistically significant.

SUPPORTING INFORMATION

Tables 1, 2; figures 1, 2, 3; crystallographic data and structure of compound **1d**; spectroscopic data (IR, ^{1}H, ^{13}C NMR) and elemental analysis for compounds **7c,e, 8c,e, 9c,e, 10c,e** and **3c,e**; References.

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ACKNOWLEDGMENT

This work was financially supported by Ministero dell'Istruzione dell'Università e della Ricerca (MIUR).

This study was performed on behalf of the EORTC-PAMM group.

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