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Original article

Synthesis, cytotoxicity, and inhibitory effects on tubulin polymerization of a new 3-heterocyclo substituted 2-styrylquinazolinones

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Abstract

In order to study the influence of 3-substitution on the cytotoxic activity of 2-styrylquinazolinones, new 6-chloro-2-styryl-3-(heteroaryl)-4(3H)-quinazolinones were synthesized by refluxing equimolar amounts of 6-chloro-2-methyl-3-(heteroaryl)-4(3H)-quinazolinones and benzaldehyde in glacial acetic acid. At 1 μg ml^{-1} concentration, almost all 2-styrylquinazolinones showed some cytotoxic activity against the L1210 and K562 leukemia cell lines. However, only 6-chloro-2-styryl-3-(pyrimidin-2yl)-4(3H)-quinazolinone inhibited the growth of these cells by over 50%. This last compound was also the only member of the series that inhibited tubulin polymerization, with an IC_{50} value of 5.8 versus 3.2 μM for colchicine. It was also examined for effects on the growth of human MCF7 breast carcinoma cells and Burkitt lymphoma CA46 cells, which had IC_{50} values of 0.34 and 1.0 μM, respectively. At 10 μM 6-chloro-2-styryl-3-(pyrimidin-2yl)-4(3H)-quinazolinone induced G2/M arrest (66%) in Burkitt cells, with a mitotic index of 20%. At 3.4 μM, it caused disruption of the cellular microtubule system of the MCF7 cells. Both these cellular effects are consistent with its mechanism of action resulting from its inhibitory effect on tubulin assembly.

Keywords: 2-Styrylquinazolinones; Antimitotic agents; Cytotoxic activity; Microtubules

1. Introduction

Inhibition of tubulin polymerization is the target of many antitumoral agents known as antimitotic agents or spindle poisons. Colchicine [1] (**I**), 2,3,4-trimethoxy-4′-carbomethoxy-1,1′-diphenyl (TBC) [2] (**II**), podophyllotoxin [3] (**III**), and combretastatin A-4 [4] (**IV**) are representative examples of compounds that inhibit microtubule assembly by binding to tubulin. All these compounds share a common structural feature, two aromatic rings directly bonded or separated by one to four carbon atoms, in such a way that they are close in space but not coplanar [5].

2-Aryl- and 2-styrylquinazolin-4(3H)-ones (**Va, b**) are compounds that possess this common structural feature for an effective interaction with tubulin. These compounds are active as antimitotic agents and inhibit tubulin polymerization [6–8]. In particular, for 2-styrylquinazolinones (**Vb**), the inhibitory activity was retained only if an intact 2-styrylquinazolinone structure was present. Activity was further enhanced by a small hydrophobic substituent at the 6-position. In contrast, inhibitory effects on both cell growth and tubulin polymerization were lost if derivatives **Vb** were substituted at the 3-position with an amino group [8].

Previously we reported [9] the synthesis and antifungal activity of 3-(3-methyl-5-isoxazolyl)-2-styrylquinazolin-4(3H)-ones (**VI**). Because of the cytotoxic activity described for styrylquinazolinones (**V**) [6], we tested some of compounds **VI** for cytotoxic activity against L1210 murine leukemia and K562 human chronic myeloid leukemia cells. Only 3-(3-methyl-5-isoxazolyl)-2-(4-chlorostyryl)-quinazolin-4(3H)-one (**VIa**) and 7-chloro-3-(3-methyl-5-isoxazolyl)-2-(2-nitrostyryl)-quinazolin-4(3H)-one (**VIb**) showed moderate cytotoxicity at 1 μg ml^{-1} (**VIa** showed 35.4% inhibition against K562 cells and **VIb** 19% inhibition against L1210 cells).

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In an effort to enhance the cytotoxic activity of this class of compounds we decided to investigate the effect of heterocyclic substitution at position 3. We synthesized and evaluated the new 6-chloro-2-styrylquinazolinones (**3b–m**), which bear such substituents as 2-pyridinyl, 2-picolinyl, 3-methyl-5-isoxazolyl, 5-methyl-3-isoxazolyl, 3-indazolyl, 5-indazolyl, 2-pyrimidinyl, 1-phenyl-3-methyl-5-pyrazolyl, and 1-ethyl-5-pyrazolyl moieties.

All the new compounds were tested for their antineoplastic activity in vitro against the L1210 and K562 cell lines. They were also evaluated for potential inhibitory effects on tubulin assembly. Only the most cytotoxic member of the group, compound **3i**, inhibited tubulin polymerization, and this compound was also examined for cellular effects on microtubules in several additional cell lines.

2. Chemistry

6-Chloro-3-phenyl-2-styryl-quinazolin-4(3H)-one (**3a**) is known [10].

3-Heterocyclo-2-styrylquinazolinones (**3b–m**) were obtained starting from 3-heterocyclo-2-methylquinazolinones (**1b–m**) by condensation with benzaldehyde (**2**) (Scheme 1).

The reaction was performed by refluxing equimolar amounts of 2-methylquinazolinones (**1b–m**) and benzaldehyde (**2**) in glacial acetic acid. The structures of the new compounds were elucidated by analytical and spectroscopic measurements. In particular, ^{1}H-NMR spectra of compounds **3** are consistent with a *trans*-olefinic structure [11]. The β-olefinic protons appeared as doublets at 6.09–6.80 δ (J = 15 Hz as required for a *trans* structure), while the α-olefinic hydrogens were found along with aromatic multiplets because of the deshielding effects of two quinazolinone nitrogens.

6-Chloro-2-methylquinazolinones **1a** [12], **1b** [13], **1c** [14], **1d** [15], **1e** [16], **1f** [17], **1g** [18], **1i** [19] and **1l** [20] were known. Compounds **1h, m** were obtained by fusion of 6-chloro-2-methyl-benzoxazin-4(3H)-one (**5**) [21] with the amino derivatives **5h, m** according to Scheme 2.

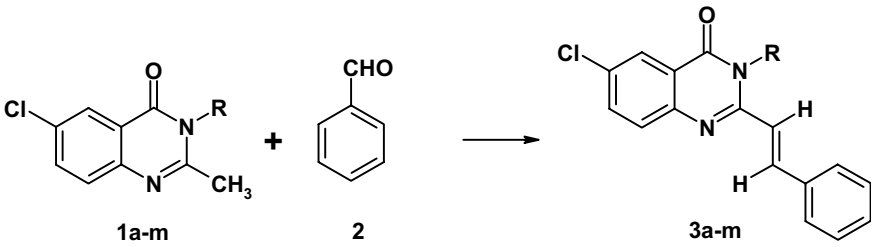
3. Pharmacology

Initially compounds **3a–m** were tested for their cytotoxicity in vitro against L1210 murine leukemia and K562 human chronic myelogenous leukemia cells by evaluating cell viability following staining with trypan blue. Antitubulin activity was evaluated by examining the effects of all compounds except **3f**, which was unavailable, as inhibitors of tubulin assembly [22]. The tubulin used was purified from bovine brain tissue [23]. Colchicine was used as a control drug in both assays.

The most active compound in the trypan blue assay was **3i**, which was also the only compound with significant activity as an inhibitor of tubulin assembly. The effects of **3i** on the growth of PtK2 *Potorus tridactylis* kidney epithelial cells and MCF7 human breast cancer cells were therefore determined, using the sulforhodamine B assay for quantitation of protein as a measure of cell growth [24]. We then examined the microtubule pattern in these two cell lines by immunofluorescence following treatment with **3i** [25]. Finally, we confirmed that **3i** was acting at the microtubule level by demonstrating that Burkitt lymphoma CA46 cells arrested at the G2/M phase of the cell cycle, with an increase in the mitotic index.

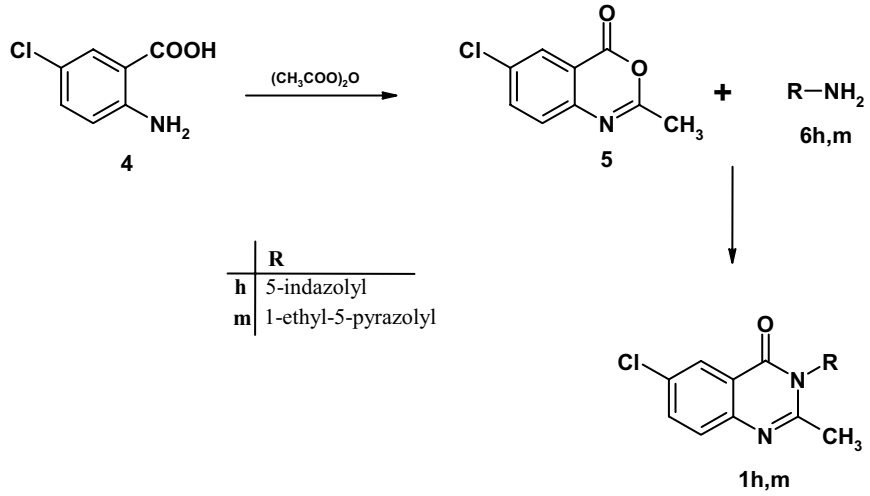
4. Results

Table 1 summarizes the growth inhibition observed when L1210 and K562 cells were treated with compounds **3a–m**



	R
a	Phenyl
b	2-Pyridinyl
c	3-Methyl-2-pyridinyl
d	3-Methyl-5-isoxazolyl
e	5-Methyl-3-isoxazolyl
f	3-Phenyl-5-isoxazolyl
g	3-Indazolyl
h	5-Indazolyl
i	2-Pyrimidinyl
l	1-Phenyl-3-methyl-5-pirazolyl
m	1-Ethyl-5-pyrazolyl

Scheme 1



Scheme 2.

and colchicine at 1 μg ml^{-1}. Some inhibitory effect was observed with most of the new compounds, but only **3i** and colchicine reduced the number of viable cells by over 50%. Definitive IC_{50} values were then determined for **3i** in the two cell lines, with values of 0.23 μg ml^{-1} (0.64 μM) and 0.38 μg ml^{-1} (1.05 μM) obtained for the L1210 and K562 cells, respectively. In both cell lines colchicine had an IC_{50} value lower than 0.01 μg ml^{-1} (0.025 μM).

The effects of compounds **3a–e, 3g–i, 3l**, and **3m** on the assembly of purified bovine brain tubulin were evaluated as described previously [22] and compared with the effect of colchicine. Except for **3i** and colchicine, no significant inhibitory effects were observed (IC_{50} values >40 μM). The IC_{50} values for **3i** and colchicine were 5.8 ± 0.2 and 3.2 ± 0.4 μM, respectively. Progressive inhibition by increasing concentrations of **3i** is shown in Fig. 1. Thus **3i**, like the previously studied styrylquinazolinones [6–8], appears to exert its inhibitory effect on cell growth through an interaction with tubulin.

Table 1
Percent growth inhibition recorded on L1210 and K562 cell lines at 1 μg ml^{-1} concentration of **3a–m** compounds

Compound	L1210	K562
3a	ns	ns
3b	ns	25.6
3c	ns	ns
3d	39.4	42.9
3e	20.2	ns
3f	43.4	23.9
3g	ns	14.5
3h	28.9	ns
3i	72.6	66.4
3l	15.3	31.0
3m	12.0	ns
Colchicine	79.0	84.7

Values are the mean of at least three independent determinations; variation was less than 15%; ns, not significant, because less than 10%.

Further cellular studies were performed to confirm this conclusion. Compound **3i** was first studied in *P. tridactylis* PtK2 kidney epithelial cells. Although no definitive IC_{50} value could be obtained, many of the cells treated with 50 μM **3i** showed a substantial loss of microtubules and developed a rounded shape (Fig. 2B) as compared with untreated cells (Fig. 2A). However, other cells in the treated culture more closely resembled untreated cells and retained a complex microtubule network (Fig. 2C).

We, therefore, also examined the effects of **3i** on MCF7 human breast cancer cells. MCF7 cells were relatively sensitive to the agent (IC_{50} value, 0.34 μM), and a larger proportion of MCF7 cells treated with 3.4 μM **3i** (compare Fig. 3B with untreated cells shown in Fig. 3A), as compared with the PtK1 cells, showed extensive disruption of their microtubule networks. In addition, a few MCF7 cells showed persistence of microtubules (Fig. 3B, cell near center of figure), although the pattern differed from that observed in untreated cells. We

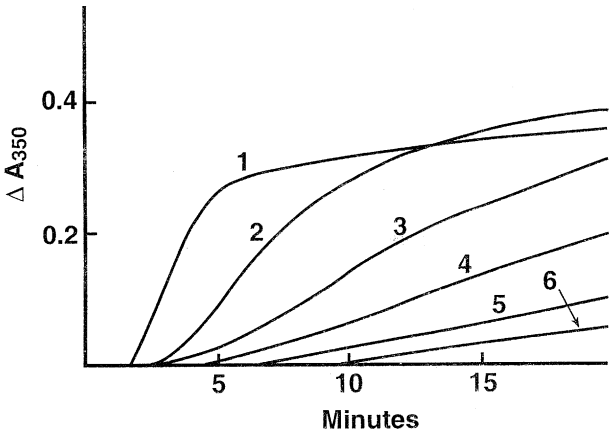


Fig. 1. Progressive inhibition of tubulin assembly by increasing concentrations of compound **3i**. Each 0.25 ml reaction mixture contained 1.0 mg ml^{-1} (10 μM) tubulin, 0.8 M monosodium glutamate (pH 6.6 with HCl in 2 M stock solution), 0.4 mM GTP, 4% (v/v) dimethyl sulfoxide, and the following concentrations of compound **3i**: curve 1, none; curve 2, 2 μM; curve 3, 4 μM; curve 4, 6 μM; curve 5, 8 μM; curve 6, 10 μM.

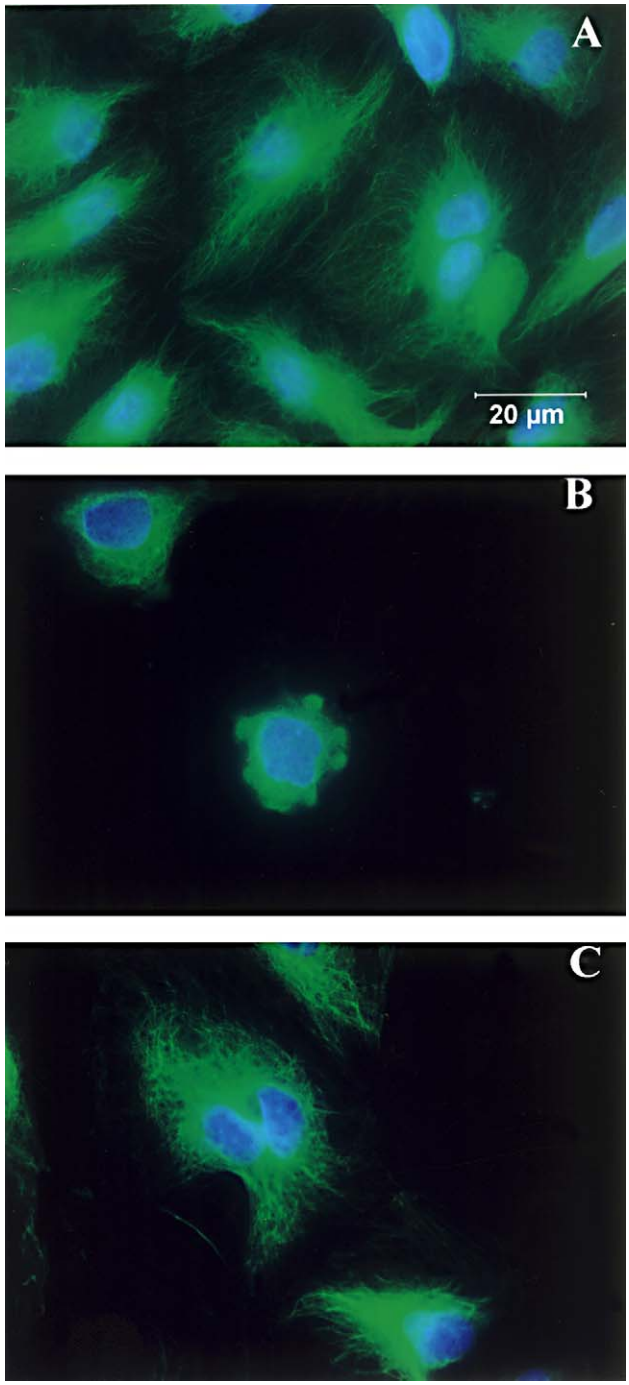


Fig. 2. Effect of compound **3i** on the microtubule network of PtK2 cells. A, untreated PtK2 cells. B and C, PtK2 cells treated with **3i** at 50 μM. Microtubules and unassembled tubulin are shown in green. DNA, stained with 4,6-diamidino-2-phenylindole (DAPI), is shown in blue.

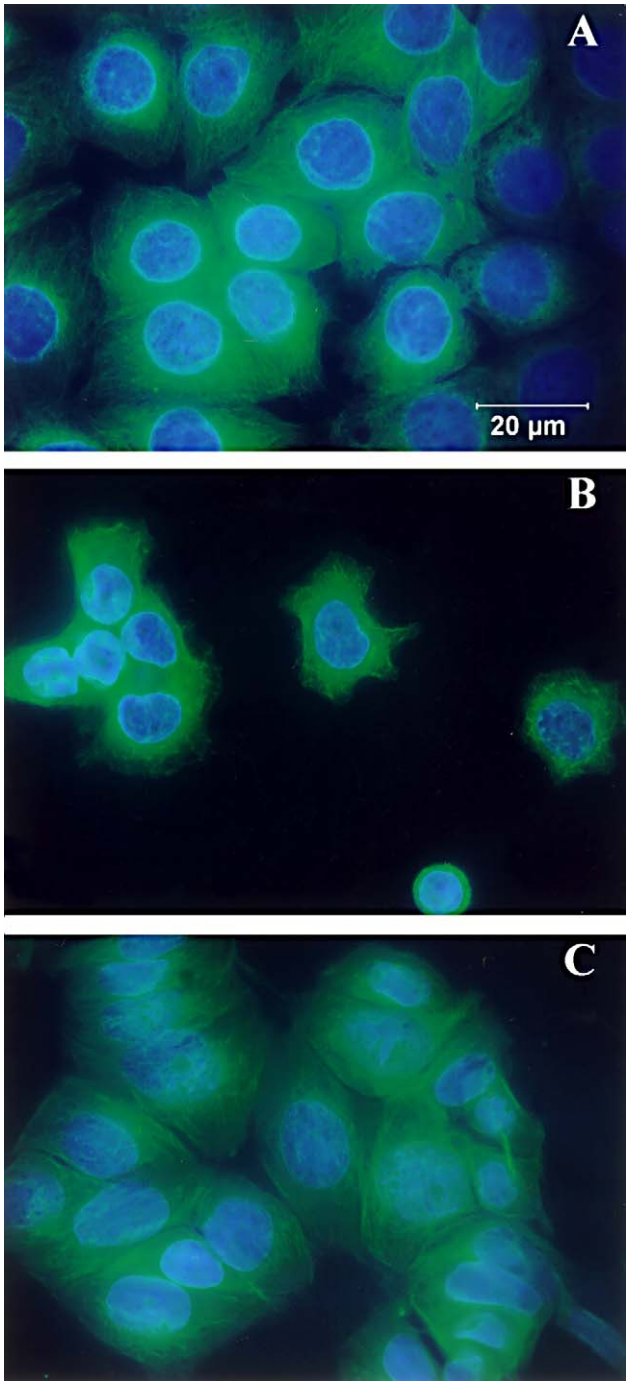


Fig. 3. Effect of compound **3i** on the microtubule network of MCF7 cells. A, untreated cells. B, MCF7 cells treated with **3i** at 3.4 μM. C, MCF7 cells treated with **3i** at 3.4 μM for 24 h and then grown another 24 h in the absence of drug. Microtubules and unassembled tubulin are shown in green. DNA, stained with DAPI, is shown in blue.

also performed a preliminary study with MCF7 cells to determine whether the morphological effects of treatment with **3i** were reversible. Following growth for 24 h in the presence of 3.4 μM **3i**, the cells were washed and grown for another 24 h in the absence of **3i**. Most of these cells had extensive microtubule networks (Fig. 3C), although the distribution pattern of the microtubules still seemed abnormal. Cells were also denser in this culture, so that it appears that cell growth had resumed.

Finally, we evaluated cell cycle effects of **3i** on human Burkitt lymphoma CA46 cells. An IC_{50} value of 1.0 μM was obtained for this cell line. When the Burkitt cells were treated with 10 μM **3i**, flow cytometry for DNA content following a 24 h treatment showed that 66% of the cells were arrested at G2/M. A morphological evaluation following a 16 h treatment showed that 20% of the cells were arrested at mitosis.

5. Discussion

Using as our scaffold 6-chloro-2-styrylquinazolin-4(3H)-one, which is one of the most active styrylquinazolinones described previously [8], we have begun to explore the effect on activity of introducing substituents at position 3. Although our best compound, **3i**, is significantly less active than the lead compound, it nonetheless has the same mechanism of action. Compound **3i** inhibits tubulin assembly, and this property causes the disappearance of microtubules in cells treated with the agent. Such treated cells arrest at the mitotic phase of the cell cycle. Comparing the data presented here with that previously published for 6-chloro-2-styrylquinazolin-4(3H)-one [8], compound **3i** is over 200-fold less active in inhibiting the growth of L1210 cells (IC_{50} value of 0.23 versus 0.001 μg ml^{-1}) but only about 50% less active as an inhibitor of tubulin polymerization (IC_{50} value of 5.8 versus 2.5 μM). This discordance indicates that it would be worthwhile examining further the effect of substitution at position 3, to identify substituents that would confer high activity in both the biochemical and cytological assays.

6. Experimental section

6.1. Chemistry

All melting points were determined on a Büchi 530 capillary melting point apparatus and are uncorrected; IR spectra were recorded with a JASCO IR-810 spectrophotometer as Nujol mull supported on NaCl disks; ^{1}H-NMR spectra were obtained using a Bruker AC-E 250 MHz spectrometer (tetramethylsilane as internal standard). Microanalyses (C, H, N) were performed in the laboratories of the Dipartimento di Scienze Farmaceutiche, Università di Catania, and were within ±0.4% of the theoretical values.

6.1.1. 6-Chloro-2-methyl-3-(heteroaryl)-quinazolin-4(3H)-ones (*1h, m*)

Equimolar amount (0.015 mol) of 6-chloro-2-methylbenzoxazinone (**5**) [21] and amines **6h, m** were heated at 160–180 °C for 2 h in an oil bath. Upon cooling, the solid reaction material was crystallized from ethanol to give pure 6-chloro-2-methyl-3-(heteroaryl)-quinazolin-4(3H)-ones (**1h, m**); yields 64–87%. Compound **1h** m.p. 118–120 °C (64%). Anal. $C_{16}H_{11}N_4OCl$ (C, H, N). IR (cm^{-1}): 3248 (NH), 1662 (CO). ^{1}H-NMR (DMSO-d_6) (δ): 2.14 (s, 3H, CH_3); 7.36–8.19 (a set of signals, 7H, aromatic protons); 13.37 (s, 1H, exchangeable NH). Compound **1m** m.p. 140 °C (87%). Anal. $C_{14}H_{13}N_4OCl$ (C, H, N). IR (cm^{-1}): 1690 (CO). ^{1}H-NMR (DMSO-d_6) (δ): 1.44 (t, 3H, CH_3); 2.28 (s, 3H, CH_3); 3.91 (q, 2H, CH_2); 6.30–8.21 (a set of signals, 5H, aromatic protons).

6.1.2. 6-Chloro-2-styryl-3-(heteroaryl)-quinazolin-4(3H)-ones (*3b–m*)

Equimolar amounts (10 mmol) of 6-chloro-2-methyl-3-(heteroaryl)-quinazolin-4(3H)-ones (**1b–m**) and benzoic aldehyde in acetic acid (10 ml) were reacted under reflux for 12 h.

The solid product that separated was filtered and crystallized from dioxane, except that in the case of compound **3c** the solid was crystallized from acetonitrile. Yield 15–42%. Compound **3b** m.p. 230–231 °C (34%). Anal. $C_{21}H_{15}N_3OCl$ (C, H, N). IR (cm^{-1}): 1683 (CO). ^{1}H-NMR (DMSO-d_6) (δ): 6.15 (d, 1H, olefinic CH, J = 15 Hz); 7.36–8.74 (a set of signals, 14H, aromatic protons and olefinic CH). Compound **3c** m.p. 260–266 °C (34%). Anal. $C_{21}H_{16}N_3OCl$ (C, H, N). IR (cm^{-1}): 1680 (CO). ^{1}H-NMR (DMSO-d_6) (δ): 2.15 (s, 3H, CH_3); 6.09 (d, 1H, olefinic CH, J = 15.2 Hz); 7.37–8.56 (a set of signals, 12H, aromatic protons and olefinic CH). Compound **3d** m.p. 235–237 °C (37%). Anal. $C_{20}H_{14}N_3O_2Cl$ (C, H, N). IR (cm^{-1}): 1703 (CO). ^{1}H-NMR (DMSO-d_6) (δ): 2.39 (s, 3H, CH_3); 6.37 (d, 1H, olefinic CH, J = 15.2 Hz); 6.81 (s, 1H, isoxazole H-4); 7.41–8.04 (a set of signals, 9H, aromatic protons and olefinic CH). Compound **3e** m.p. 227–229 °C (37%). Anal. $C_{20}H_{14}N_3O_2Cl$ (C, H, N). IR (cm^{-1}): 1700 (CO). ^{1}H-NMR (DMSO-d_6) (δ): 2.58 (s, 3H, CH_3); 6.60 (d, 1H, olefinic CH, J = 15 Hz); 6.67 (s, 1H, isoxazole H-4); 7.39–8.08 (a set of signals, 9H, aromatic protons and olefinic CH). Compound **3f** m.p. 222–223 °C (40%). Anal. $C_{23}H_{16}N_3O_2Cl$ (C, H, N). IR (cm^{-1}): 1701 (CO). ^{1}H-NMR ($CDCl_3$) (δ): 6.42 (d, 1H, olefinic CH, J = 15.3 Hz); 6.85 (s, 1H, isoxazole H-4); 7.33–8.23 (a set of signals, 14H, aromatic protons isoxazole H-4 and olefinic CH). Compound **3g** m.p. >280 °C (13%). Anal. $C_{23}H_{15}N_4OCl$ (C, H, N). IR (cm^{-1}): 3308 (NH), 1677 (CO). ^{1}H-NMR ($CDCl_3$) (δ): 6.34 (d, 1H, olefinic CH, J = 15.5 Hz); 7.12–8.34 (a set of signals, 13H, aromatic protons and olefinic CH); 11.07 (s, 1H, exchangeable NH). Compound **3h** m.p. >280 °C (38%). Anal. $C_{23}H_{15}N_4OCl$ (C, H, N). IR (cm^{-1}): 3244 (NH); 1668 (CO). ^{1}H-NMR (DMSO-d_6) (δ): 6.33 (d, 1H, olefinic CH, J = 15 Hz); 7.26–8.22 (a set of signals, 13H, aromatic protons, indazole H-3 and olefinic CH), 13.23 (s, 1H, exchangeable NH). Compound **3i** m.p. 236–238 °C (30%). Anal. $C_{20}H_{13}N_4OCl$ (C, H, N). IR (cm^{-1}): 1695 (CO). ^{1}H-NMR ($CDCl_3$) (δ): 6.09 (d, 1H, olefinic CH, J = 15.4 Hz); 7.31–9.03 (a set of signals, 12H, aromatic protons and olefinic CH). Compound **3l** m.p. 198–200 °C (77%). Anal. $C_{26}H_{19}N_4OCl$ (C, H, N). IR (cm^{-1}): 1682 (CO). ^{1}H-NMR ($CDCl_3$) (δ): 2.48 (s, 3H, CH_3); 6.38–6.45 (s + d, 2H, pyrazole H-4 and olefinic CH); 7.24–8.19 (a set of signals, 14H, aromatic protons and olefinic CH). Compound **3m** m.p. 177–178 °C (40%). Anal. $C_{21}H_{17}N_4OCl$ (C, H, N). IR (cm^{-1}): 1691 (CO). ^{1}H-NMR ($CDCl_3$) (δ): 1.40 (t, 3H, CH_3); 3.93 (q, 2H, CH_2); 6.33 (d, 1H, olefinic CH, J = 15.9 Hz); 6.37 (s, 1H, pyrazole H-4); 7.36–8.19 (a set of signals, 10H, aromatic protons, pyrazole H-3 and olefinic CH).

6.2. Biology

6.2.1. Cytotoxic activity in vitro

Compounds **3a–m** were tested for inhibitory effects on the in vitro growth of L1210 murine leukemia cells and K562 human chronic myelogenous leukemia cells. The cell lines were grown at 37 °C in a humidified atmosphere containing 5% CO_2 in RPMI-1640 medium supplemented with 10%

fetal calf serum and antibiotics. The cells were suspended at a density of 10^5 cells ml^{-1}, transferred in 1 ml aliquots to a 24-well plate, and incubated at 37 °C for 48 h without or with varying concentrations of test compounds [26]. Number of viable cells was determined by exclusion of trypan blue by counting in a hemacytometer. The IC_{50} value for **3i** was defined as the concentration that reduced the viable cell number by 50%.

The IC_{50} values for **3i** against PtK2 cells and MCF7 cells were determined by the sulforhodamine B method [24]. In brief, the cells were allowed to establish themselves for 18–24 h in the chambers of a 96-well microtitre plate, test compound(s) in varying concentrations were added, incubation at 37 °C continued for 48 h, and cellular protein was measured by adding sulforhodamine B to the plate wells. The IC_{50} value is defined as the concentration that reduces the increase in cellular protein by 50% compared to untreated cells.

The Burkitt lymphoma CA46 cells were grown in RPMI-1640 medium supplemented with 10% fetal calf serum at 37 °C in a humidified atmosphere with 5% CO_2. Following test compound addition, cells were grown for an additional 24 h. Cell number was then determined with a Coulter counter. The IC_{50} value is defined as the concentration that reduced increase in cell number by 50% relative to an untreated control culture. Cells were examined by flow cytometry for DNA content after 24 h of growth and for their mitotic index by Giemsa staining after 16 h of growth.

6.2.2. Immunofluorescence

Direct immunofluorescence was performed essentially as described previously [25]. The FITC-conjugate of anti-β-tubulin clone TUB2.1 monoclonal antibody was obtained from Sigma. A Nikon Eclipse E800 microscope, equipped with appropriate epifluorescence components and image capture software was used. Cells were examined and images obtained with the 100× oil objective (N.A. 1.30).

6.2.3. Tubulin assembly assay

Purified bovine brain tubulin was prepared as described in detail previously [23]. This tubulin was used to determine the effects of compounds **3a–m** (except **3f**) and of colchicine on polymerization, as described elsewhere [23]. In brief, tubulin and varying concentrations of test compound were preincubated for 15 min at 30 °C without GTP to permit drug to interact with protein. Reaction mixtures were chilled on ice, GTP (0.4 mM) was added, and the complete reaction mixtures were transferred to 0 °C cuvettes in a Beckman DU7400 spectrophotometer equipped with an electronic temperature controller. Baseline apparent A_{350} readings for each cuvette were determined and defined as zero readings. The temperature was jumped to 30 °C, and the turbidity change in each cuvette was monitored every 15 s by measuring absorbance at 350 nm. The IC_{50} value is defined as the concentration of test compound that reduced the extent of polymerization (increase in turbidity) by 50% after 20 min at 30 °C.

Acknowledgements

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