
Demetrio Raffa a,*, Benedetta Maggio a, Fabiana Plescia a, Stella Cascioferro a, Salvatore Plescia a, Maria Valeria Raimondi a, Giuseppe Daidone a, Manlio Tolomeo b, Stefania Grimau d b, Antonietta Di Cristina b, Rosaria Maria Pipitone b, Ruoli Bai c, Ernest Hamel c

a Dipartimento di Scienze e Tecnologie Molecolari e Biomolecolari, Via Archirafi, 32, 90123 Palermo, Italy
b Centro Interdepartimentale di Ricerca in Oncologia Clinica e Dipartimento Biochimico di Medicina Interna e Specialistica, Università di Palermo, Palermo, Italy
c Screening Technologies Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute at Frederick, National Institutes of Health, Frederick, MD 21702, USA

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A B S T R A C T
Several new 2-\{[(2E)-3-phenylprop-2-enoyl]amino\}benzamides 12a–s and 17t–v were synthesized by stirring in pyridine the (E)-3-(2-R1-3-R2-4-R3-phenyl)acrylic acid chlorides 11c–k and 11t–v with the appropriate antranilamide derivatives 10a–e or the 5-iodoanthranilic acid 13. Some of the synthesized compounds were evaluated for their in vitro antiproliferative activity against the full NCI tumor cell line panel derived from nine clinically isolated cancer types (leukemia, non-small cell lung, colon, CNS, melanoma, ovarian, renal, prostate and breast). COMPARE analysis, effects on tubulin polymerization in cells and with purified tubulin, and effects on cell cycle distribution for 17t, the most active of the series, indicate that these new antiproliferative compounds act as antitubulin agents.

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1. Introduction

During a screening program to find antiproliferative compounds in our laboratory's collection of small organic molecules, the 2-cinnamamido-5-iodobenzamide 1 was found at 10 μM to inhibit proliferation of the leukemic cell line K562 by 74%.

Compound 1 belongs to cinnamoyl anthranilates, which represent a class of biologically active substances of great importance in medicinal chemistry. Tranilast (Rizaben®) 2 is an antiallergic drug approved in 1982 for use in Japan and South Korea for bronchial asthma and was also investigated for use as an antiproliferative agent on drug-eluting stents. Its derivative (E)-2-(3-(3,4-dimethoxyphenyl)acrylamido)benzamide 3 [1] is another member of this class of compounds and is more potent than the lead compound, Tranilast (Fig. 1).

Other biological activities possessed by this class of compounds are antifibrotic and antinflammatory properties [2,3] and inhibition of cornea pterygium progression and blood vessel development [4,5]. Finally, cinnamoyl anthranilates are useful for prevention and treatment of glomerular diseases [6] and diseases caused by the excessive proliferation of vascular intimal cells [7].

However, despite their wide range of biological activities, a review of the literature revealed that no anticancer activity had been described for cinnamoyl anthranilates. Thus, the activity of compound 1 as an inhibitor of K562 proliferation led us to explore the potential of this class of compounds as anticancer agents, and we therefore synthesized a series of novel cinnamoyl anthranilates and screened the compounds for antileukemic activity. Our work enabled us to perform an initial study of their structure-activity relationships and to determine their mechanism of action. Compounds 12a–s and 17t–v were initially tested in vitro for their antileukemic activity against the K562 (human chronic myelogenous leukemia) cell line (Table 1). Among these, 12a–c and 17t,c, which showed the best antiproliferative activity, were selected by the National Cancer Institute (NCI) for evaluation in the 60 human tumor cell line screen of the NCI.

2. Results and discussion

2.1. Chemistry

A series of 2-\{[(2E)-3-phenylprop-2-enoyl]amino\}benzamides 12a–s and 17t–v was synthesized by stirring the (E)-3-(2-R1-3-R2-4-R3-phenyl)acrylic acid chlorides 11c–k with the appropriate antranilamide derivatives 10a–e or the 5-iodoanthranilic acid 13.
starting materials, 2-cinnamamido-5-iodobenzoic acids 15t–v, were obtained by stirring the 5-idoanthranilic acid 13 and the cinnamoyl chlorides 11t–v. The reaction gave a mixture of (E)-6-iodo-2-styryl-4H-benzof[d]1,3]oxazin-4-ones 14t–u and 2-cinnamamido-5-iodobenzoic acids 15t–u, and the mixtures were treated with aqueous Na3PO4 [9] to give the corresponding acids 15t [10], 15u,v as the only products. By treating the acids 15t–v with ethyl chloroformate, the 2H-3,1-benzoxazine-2,4-diones 16t–v were obtained [11], which, in turn, converted to the 2-[(2E)-3-phenylprop-2-enoyl]amino]benzamides 17t–v by refluxing them in an aqueous ammonia solution [8].

The structures of the new compounds were determined by analytical and spectroscopic measurements. In particular, 1H NMR spectra of compounds 12a–s and 17u–t were consistent with an E-olefinic structure. They showed signals attributable to the β-olefinic protons at 6.66–6.97 δ with coupling constants of 16.6–14.7 Hz, as required for E-structures [12], while the α-olefinic hydrogens were found along with aromatic multiplets. Moreover, their 1H NMR spectra showed both the NH and NH2 amide signals; the cinnamamido NH proton appeared as a singlet at 11.77–11.92 δ, while, according to the literature [13], the presence of an intramolecular hydrogen bond renders the benzamido NH2 protons diastereotopic. H4 is easily exchangeable with D2O and appeared as a singlet at 8.24–8.44 δ. H5 was found at a lower field along with the aromatic multiplets.

2.2. Biology

Synthesized 2-[(2E)-3-phenylprop-2-enoyl]amino]benzamides 12a–s and 17u–t were initially tested in vitro for their antileukemic activity against the K562 (human chronic myelogenous leukemia) cell line. Colchicine 18, whose antileukemic activity is well known, and the 2-cinnamamidobenzamides 4 [13] (Fig. 1), were used as reference compounds. The percent growth inhibition at a screening concentration of 10 μM and the IC50 values for compounds that exhibited at least 50% of growth inhibition at 10 μM are shown in Table 1. Compounds 12a–s and 17u–t had inhibitory activity against the K562 cells ranging from 22.0 to 74.5% at 10 μM, with 12a–d, 12k,l, and 17u IC50 (0.57–8.1 μM) being the most active compounds. Our data (Table 1) showed positive effects following substitution with halogens at the 5 position of the benzamido moiety; the substitution was present only on the benzamido moiety (compounds 17u and 17u). As far as structure–activity relationships are concerned, it seems that the introduction of a substituent in both the benzamido and styryl moieties is favorable for inhibition of K562 cell growth relative to the unsubstituted 2-cinnamamidobenzamide 4 (Table 1). However, the best activity was obtained when the substitutions were present only on the benzamido moiety (compounds 17u and 17u). Compounds substituted in both the benzamido and styryl moieties (compounds 12e–s, 17v) were less active, even if antiproliferative activity was maintained in the ortho-styryl derivatives (12d,l,l,1,u).

Compounds 12a, 12b, 12c, 17t, and 17u were evaluated by the NCI for testing against a panel of approximately 60 human cell lines derived from seven clinically isolated cancer types (lung, colon, melanoma, renal, ovarian, brain, and leukemia) according to the NCI standard protocol [14] (Table 2). The data summarized in Table 2 showed that 12a, 12b, 12c, 17t, and 17u caused 50% growth inhibition at micromolar (12a, 12b, 12c) and submicromolar concentrations (17t, 17u) against every type of tumor cell line investigated.

Moreover, a mean graph midpoint (MG_MID) is calculated for the GI50, TGI and LC50 parameters, giving an average activity parameter for all the cell lines. For the calculation of the MG_MID, insensitive cell lines are included and assigned as their values the highest concentration tested. Considering the MG_MID values
The most active compound of the series was derivative 17t, at both the GI50 and TGI levels, followed by 17u and 12c. Compound 17t was selected by the NCI for evaluation in its vivo toxicity assay, with the finding that the compound was nontoxic at a dose of 400 mg/kg in nontumored mice. Compound 17t was also selected by the NCI for testing in the hollow fiber assay, a preliminary in vivo screening tool, but the compound was not sufficiently active for evaluation in xenograft models.

To predict the probable mechanism of action, the NCI’s bioinformatic tool COMPARE analysis [15] was performed for the most active compound 17t. When tested as seed against the NCI “standard agents” Database, the compound showed Pearson Correlation Coefficients (PCC) of 0.492 and 0.476 at the GI50 level and higher values, 0.677, 0.600, 0.597, at the TGI level. In all cases the highest PCC’s were with compounds NSC 332598 (rhizoxin), NSC 125973 (paclitaxel), NSC 49842 (vinblastine sulfate) and NSC 153858 (maytansine), which are all antimitotic agents directed against tubulin.

To verify the prediction of the COMPARE algorithm, the effects of 17t on cell cycle distribution as determined by flow cytometry and on tubulin polymerization were evaluated.

As shown in Fig. 2, 17t caused a dose dependant increase of K562 cells in the G2-M phase of the cell cycle and a decrease of cells in G0-G1 after a 24 h treatment. This is the typical flow cytometric cell cycle distribution observed with drugs targeting tubulin (see panel b in Fig. 2), which is the main component of the mitotic spindle.

After 48 h 17t caused extensive apoptosis in K562 cells, with an AC50 (concentration inducing 50% apoptosis) of 3.7 μM (Fig. 3). Apoptosis in this experiment was measured as described in the Experimental Section.

To confirm the ability of 17t to act on microtubules, the percentage of cells blocked in mitosis (M) was morphologically determined after staining cells with ethidium bromide and acridine orange. The percentage of K562 cells blocked in the M phase after 24 h of exposure to 1 μM 17t was 35 ± 6 (Fig. 4A, untreated K562 cell as control < 1%). Of interest, concentrations of 17t higher than 2 μM induced morphologic alterations in almost all treated cells, suggesting an interaction between 17t and the cytoskeleton of K562 cells (Fig. 4C and D).

To examine the effects of 17t on microtubules (tubulin) and microfilaments (actin), PtK2 cells were examined by direct immunofluorescence. These cells were selected for this examination since their flattened morphology yields high quality images of cytoskeletal elements, particularly the microtubule and microfilament networks. As shown in Fig. 5, the microtubules disappeared while the microfilaments persisted with micromolar 17t. In addition, we found that 17t partially inhibited the polymerization of purified tubulin, but this effect was relatively weak, as shown in Fig. 6, with a comparison to the much stronger inhibition observed with colchicine 18. With 17t, we observed a concentration dependend inhibition of the rate of microtubule assembly, beginning at about 10 μM. However, within the concentration range we were able to examine (up to 40 μM), there was no effect on the extent of assembly. It should be pointed out that 17t appeared to partially precipitate at 40 μM. With the classic antitubulin agent colchicine 18, the rate of assembly was 50% inhibited at about 2 μM, and the extent of assembly at about 5 μM. At 7 and 10 μM colchicine 18, tubulin polymerization was essentially completely inhibited, an effect that we were unable to achieve with 17t.

Taken together, our data suggest that 17t interacts with tubulin, preventing formation of the mitotic spindle and thereby caused

**Scheme 1.** Reagents and conditions: (a) SOCl2, reflux, 5 h; (b) acetonitrile, aqueous ammonia (25%), reflux, 8 h; (c) aqueous ammonia (25%), stirring, rt, 1 h; (d) SnCl2, HCl (35%), stirring, 0–5 °C, (24 h); (e) pyridine, stirring, rt, 24 h.
the block in M phase. Inhibition of tubulin assembly would also disrupt the integrity of the microtubule cytoskeleton in interphase cells, causing extensive alterations in cell morphology. Although its effect on in vitro tubulin assembly is relatively weak, the cellular effects obtained with 17t are most consistent with those observed with more potent antitubulin agents. Furthermore, disruption of cellular microtubules invariably results in activation of cellular apoptosis.

3. Conclusions

The data reported here show that the 2-[[2E]-3-phenylprop-2-enoylamino]benzamides 12a–s and 17t–v caused growth inhibition against many tumor cell lines. The best agents inhibited proliferation at low micromolar (12a, 12b, 12c) and submicromolar concentrations (17t, 17u) against every tumor cell line investigated. The best activity was obtained when the 5 position of the benzoamide moiety was substituted with an iodine atom. COMPARE analysis, effects on tubulin polymerization in cells and with purified tubulin, and effects on cell cycle distribution, including induction of apoptosis, indicate that these new antiproliferative compounds act as antitubulin agents.

4. Experimental

4.1. Chemistry

4.1.1. General

Reaction progress was monitored by TLC on silica gel plates (Merck 60, F254, 0.2 mm). Organic solutions were dried over Na2SO4. Evaporation refers to the removal of solvent on a rotary evaporator under reduced pressure. All melting points were determined on a Büchi 530 capillary melting point apparatus and are uncorrected. IR spectra were recorded with a Perkin Elmer Spectrum RXI FT-IR System spectrophotometer, with compound as a solid in a KBr disc. 1H NMR spectra were obtained using a Bruker AC-E 300 MHz spectrometer (tetramethylsilane as internal standard): chemical shifts are expressed in δ values (ppm). Merck silica gel (Kiesegel 60/230–400 mesh) was used for flash chromatography columns. Microanalyses data (C, H, N) were obtained by an Elemental Vario EL III apparatus and were within ±0.4% of the theoretical values. Yields refer to purified products and are not optimized. The names of the products were obtained using the ACD/I-Lab Web service (ACD/IUPAC Name Free 8.05).
4.1.2. General procedure for preparation of 5-methyl-2-nitrobenzyl chloride 6a and 3-phenacylloyl chloride 11c–k

Substituted benzoyl and acryloyl chlorides 6a, 11c–k and 11v were obtained by refluxing for 5 h the appropriate acid derivatives 5a, 8c–k and 8t–v (0.01 mol) with thionyl chloride (7.25 ml) [16]. After evaporation under reduced pressure, the crude liquid residue was used for subsequent reactions without purification.

4.1.3. Preparation of 5-methyl-2-nitrobenzamide 7a

To 0.01 mol of 5-methyl-2-nitrobenzyl chloride 6a 10 ml of aqueous ammonia solution (25%) and 33 ml of acetonitrile were added. The solution was first refluxed for 8 h, then evaporated under reduced pressure to give pure 7a.

4.1.4. Preparation of 5-methyl-2-aminobenzamide 10a

To a magnetically stirred suspension of stannous chloride 0.038 mol in concentrated HCl (37%) (15 ml), 0.013 mole of 3-phenylacryloyl chloride 11c was added at a rate so that the temperature of the slurry was maintained below 5 °C (about 1 h). After addition was complete, the mixture was stirred for 24 h. The white slurry thus obtained was filtered, washed with cold water (150 ml), and aqueous sodium hydroxide (40%) was added until the tin salt dissolved. The solution was then extracted with ethyl acetate (3 × 150 ml), and the dried and evaporated in vacuo to obtain pure 10a.

4.1.5. General procedure for preparation of aminobenzamides 10b,c

A mixture of 0.01 mol of 2H-3,1-benzoxazine-2,4(1H)-diones 9b,c and 25 ml of aqueous ammonia solution (25%) was stirred for 1 h. The precipitate was removed by filtration, washed with aqueous ammonia solution (5%) and crystallized from ethanol.


To a cold (0–5 °C) stirred suspension of aminobenzamides 10a–c (0.016 mol) in pyridine (13 ml), 0.016 mol of the appropriate 3-phenacylloyl chloride 11c–k was added over 30 min. After addition was complete, the solution was stirred for 24 h and then poured onto crushed ice. The precipitate was removed by filtration, washed with water, and crystallized from ethanol.

5-Methyl-2-[(2E)-3-phenylprop-2-enoyl]amino]benzamide (12a): yield 85%; mp 228–230 °C (dioxane); i.R. (KBr) cm⁻¹ 3400-3258 (NH, NH₂), 1681, 1651 (2XCO); ᶦ H NMR (DMSO) δ 2.31 (s, 3H, CH₃); 6.89 (d, 1H, J = 15.6 Hz, olefinic CH); 7.32–8.49 (a set of signals, 9H, aromatic protons and NH-H); 7.58 (d, 1H, J = 15.6 Hz, olefinic CH); 8.24 (s, 1H, NH–H, exchangeable); 11.80 (s, 1H, NH, exchangeable). Anal. (C₁₇H₁₆N₂O₂) C,H,N.

5-Chloro-2-[(2E)-3-phenylprop-2-enoyl]amino]benzamide (12b): yield 67%; mp 218–220 °C (dioxane); i.R. (KBr) cm⁻¹ 3384, 3218 (NH, NH₂), 1682, 1659 (2XCO); ᶦ H NMR (DMSO) δ 6.83 (d, 1H, J = 14.9 Hz, olefinic CH); 7.43–8.63 (a set of signals, 10H, aromatic protons, olefinic CH and NH–H); 8.42 (s, 1H, NH–H, exchangeable); 11.82 (s, 1H, NH, exchangeable). Anal. (C₁₇H₁₃ClNO₂) C,H,N.

5-Bromo-2-[(2E)-3-phenylprop-2-enoyl]amino]benzamide (12c): yield 98%; mp 229–231 °C (dioxane); i.R. (KBr) cm⁻¹ 3388, 3217 (NH, NH₂), 1682, 1659 (2XCO); ᶦ H NMR (DMSO) δ 6.82 (d, 1H, J = 14.9 Hz, olefinic CH); 7.43–8.58 (a set of signals, 10H, aromatic protons, olefinic CH and NH–H); 8.44 (s, 1H, NH–H, exchangeable); 11.84 (s, 1H, NH, exchangeable). Anal. (C₁₇H₁₃BrNO₂) C,H,N.

5-Chloro-2-[(2E)-3-(3-chlorophenyl)prop-2-enoyl]amino]benzamides (12d): yield 36%; mp 268–270 °C (dioxane); i.R. (KBr) cm⁻¹ 3365, 3155 (NH, NH₂), 1686, 1661 (2XCO); ᶦ H NMR (DMSO) δ 6.90 (d, 1H, J = 15.2 Hz olefinic CH); 7.38–8.63 (a set of signals, 9H, aromatic protons, olefinic CH and NH–H); 8.43 (s, 1H, NH–H, exchangeable); 11.92 (s, 1H, NH, exchangeable). Anal. (C₁₇H₁₃Cl₂NO₂) C,H,N.

5-Chloro-2-[(2E)-3-(3-chlorophenyl)prop-2-enoyl]amino]benzamides (12e): yield 88%; mp 260–261 °C (dioxane); i.R. (KBr) cm⁻¹ 3351, 3157 (NH, NH₂), 1681, 1662 (2XCO); ᶦ H NMR (DMSO) δ 6.97 (d, 1H, J = 16.6 Hz olefinic CH); 7.45–8.63 (a set of signals, 9H, aromatic protons, olefinic CH and NH–H); 8.42 (s, 1H, NH–H, exchangeable); 11.79 (s, 1H, NH, exchangeable). Anal. (C₁₇H₁₃Cl₂NO₂) C,H,N.
The highest tested concentration was used for the calculation.

... with 1 antimitotic drug used as internal standard (60 nM taxol (b)) and with 1...

... used as internal standard (60 nM taxol (b)) and with 1...

... the standard propidium iodide procedure as described in Materials and Methods. Sub-G0 ...

... the sub-G0 population was determined by flow cytometry. Apoptosis was evaluated after 48 h of...

- 5-Chloro-2-[(2E)-3-(4-chlorophenyl)prop-2-enoyl]lamino]benzamides (12f): yield 58%; mp 243–244 °C (dioxane); I.R. (KBr) cm⁻¹ 3356, 3283, 3177 (NH, NH₂), 1675, 1661 (2XCO); 1H NMR (DMSO) δ 6.87 (d, 1H, J = 14.9 Hz, olefinic CH); 7.47–8.62 (a set of signals, 9H, aromatic protons, olefinic CH and NH–H); 8.43 (s, 1H, NH–H, exchangeable); 11.82 (s, 1H, NH, exchangeable). Anal. (C_{16}H_{12}ClN₂O₂) C, H, N

- 2-[(2E)-3-(2-bromophenyl)prop-2-enoyl]lamino]benzamides (12g): yield 66%; mp 261–262 °C (dioxane); I.R. (KBr) cm⁻¹ 3366, 3157 (NH, NH₂), 1686, 1662 (2XCO); 1H NMR (DMSO) δ 6.87 (d, 1H, J = 14.8 Hz, olefinic CH); 7.35–8.63 (a set of signals, 9H, aromatic protons, olefinic CH and NH–H); 8.43 (s, 1H, NH–H, exchangeable); 11.90 (s, 1H, NH, exchangeable). Anal. (C_{16}H_{12}BrClN₂O₂) C, H, N

Table 3
Overview of the results of the in vitro antitumor screening for compounds 12a–c and 17t,u.

<table>
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<tr>
<th>Comp</th>
<th>no. Studied</th>
<th>pGI50</th>
<th>range</th>
<th>MG_MID</th>
<th>pGI50</th>
<th>range</th>
<th>MG_MID</th>
<th>pLC50</th>
<th>range</th>
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<td>5.36</td>
<td>30</td>
<td>5.3–4.00</td>
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</tr>
<tr>
<td>12b</td>
<td>58</td>
<td>5.65–4.82</td>
<td>6.65</td>
<td>29</td>
<td>6.06–4.00</td>
<td>4.53</td>
<td>7</td>
<td>5.19–4.00</td>
<td>4.06</td>
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</tr>
<tr>
<td>12c</td>
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<td>6.64–5.47</td>
<td>5.99</td>
<td>23</td>
<td>6.18–4.00</td>
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<td>10</td>
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<td>5.97</td>
<td>44</td>
<td>6.41–4.00</td>
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<td>17</td>
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<td>5.97</td>
<td>32</td>
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<td>8</td>
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<td>4.05</td>
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</tr>
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</table>

Compounds were studied in vitro in the European Institute of Oncology disease-oriented human tumor cell screen.

... the number of cell lines.

Fig. 2. Effects of compound 17t on DNA content/cell following treatment of K562 cells for 24 h. The cells were cultured without compound (control, a), with an antimitotic drug used as internal standard (60 nM taxol (b)) and with 1 µM (c), 2 µM (d), or 4 µM (e) 17t. Cell cycle distribution was analyzed by the standard propidium iodide procedure as described in Materials and Methods. Sub-G0–G1 (A), G0–G1, S, and G2-M cells are indicated in (a).

Fig. 3. Percentage of apoptosis induced by compound 17t in K562 cells. Cells were cultured with different concentrations of 17t. Apoptosis was evaluated after 48 h of treatment as described in the experimental section. Bars: ±S.E.
5-Chloro-2-{{(2E)-3-(4-methylphenyl)prop-2-enoyl}amino}benzamides (12k): yield 82%; mp 238–239 °C (dioxane); I.R. (KBr) cm⁻¹ 3379, 3212 (NH, NH₂), 1682, 1660 (2XCO); ¹H NMR (DMSO) δ 2.34 (s, 3H, CH₃); 6.78 (d, 1H, J = 15.2 Hz, olefinic CH); 7.23–8.62 (a set of signals, 9H, aromatic protons, olefinic CH and NH–H); 8.43 (s, 1H, NH–H, exchangeable); 11.77 (s, 1H, NH, exchangeable). Anal. (C₁₆H₁₂BrClN₂O₂) C, H, N.

5-Bromo-2-{{(2E)-3-(2-chlorophenyl)prop-2-enoyl}amino}benzamides (12l): yield 57%; mp 255–256 °C (dioxane); I.R. (KBr) cm⁻¹ 3357, 3285, 3178 (NH, NH₂), 1673, 1660 (2XCO); ¹H NMR (DMSO) δ 6.87 (d, 1H, J = 14.7 Hz, olefinic CH); 7.47–8.55 (a set of signals, 9H, aromatic protons, olefinic CH and NH–H); 8.42 (s, 1H, NH–H, exchangeable); 11.79 (s, 1H, NH, exchangeable). Anal. (C₁₆H₁₂BrClN₂O₂) C, H, N.

5-Chloro-2-{{(2E)-3-(4-methylphenyl)prop-2-enoyl}amino}benzamides (12n): yield 81%; mp 258–259 °C (dioxane); I.R. (KBr) cm⁻¹ 3357, 3285, 3178 (NH, NH₂), 1673, 1660 (2XCO); ¹H NMR (DMSO) δ 2.34 (s, 3H, CH₃); 6.78 (d, 1H, J = 15.2 Hz, olefinic CH); 7.23–8.62 (a set of signals, 9H, aromatic protons, olefinic CH and NH–H); 8.43 (s, 1H, NH–H, exchangeable); 11.77 (s, 1H, NH, exchangeable). Anal. (C₁₆H₁₂BrClN₂O₂) C, H, N.

5-Bromo-2-{{(2E)-3-(2-methylphenyl)prop-2-enoyl}amino}benzamides (12p): yield 98%; mp 238–240 °C (dioxane); I.R. (KBr) cm⁻¹ 3357, 3285, 3178 (NH, NH₂), 1673, 1660 (2XCO); ¹H NMR (DMSO) δ 2.32 (s, 3H, CH₃); 6.74 (d, 1H, J = 15.4 Hz, olefinic CH); 7.25–8.56 (a set of signals, 9H, aromatic protons, olefinic CH and NH–H); 8.42 (s, 1H, NH–H, exchangeable); 11.89 (s, 1H, NH, exchangeable). Anal. (C₁₅H₁₂BrN₂O₂) C, H, N.

5-Bromo-2-{{(2E)-3-(3-bromophenyl)prop-2-enoyl}amino}benzamides (12q): yield 88%; mp 236–237 °C (dioxane); I.R. (KBr) cm⁻¹ 3357, 3285, 3178 (NH, NH₂), 1673, 1660 (2XCO); ¹H NMR (DMSO) δ 2.34 (s, 3H, CH₃); 6.80 (d, 1H, J = 16.5 Hz, olefinic CH); 7.24–8.63 (a set of signals, 9H, aromatic protons, olefinic CH and NH–H); 8.42 (s, 1H, NH–H, exchangeable); 11.80 (s, 1H, NH, exchangeable). Anal. (C₁₅H₁₂BrN₂O₂) C, H, N.
signals, 9H, aromatic protons, olefinic CH and NH; 8.42 (s, 1H, NH, exchangeable); 11.78 (s, 1H, NH, exchangeable). Anal. (C₁₇H₁₅BrN₂O₂) C, H, N.

4.1.7. General procedure for preparation of 2-cinnamamido-5-iodobenzoic acids 15t–v

To an ice cooled (0–5 °C) stirred solution of 2-amino-5-iodobenzoic acid 13 (3 mmol) in anhydrous pyridine (20 ml) 2 mmol of cinnamoyl chlorides 11t–v was added. The solution was left stirring overnight, then poured into cold water. The precipitate was collected as a mixture of (E)-6-iodo-2-styryl-4H-benzo[d][1,3]oxazin-4-ones 14t–u and 2-cinnamamido-5-iodobenzoic acids 15t–v. The mixture was refluxed in 0.01 M aqueous Na₃PO₄ for 20 h. The solution was allowed to cool to room temperature and, after filtration, was acidified with HCl (0.1 M) to pH 2 to give the corresponding acids 15t [10] and 15u as the only products. Finally, the precipitate was removed by filtration and washed with cold chloroform to obtain pure 15t,u. In the case of cinnamoyl chloride 11v, treatment with 2-amino-5-iodobenzoic acid 13 (3 mmol) in anhydrous pyridine (20 ml) directly gave pure (E)-5-iodo-2-(3-o-tolylacrylamido)benzoic acid 15v.

(E)-2-(3-(2-chloropheny)acrylamido)-5-iodobenzoic acid (15u): yield 80%; mp 250–252 °C (ethanol); I.R. (KBr) cm⁻¹ 3448, 3117 (NH, OH), 1703, 1688 (2XCO); ¹H NMR (DMSO) δ 6.94 (d, 1H,
and was used as is without purification. Yield 83%; mp 201 °C (dioxane). After this time, acetyl chloride (1.5 ml) was added, and reprecipitated, was removed by filtration and crystallized from dioxane.

Baseline were established, and the temperature was jumped to 30 °C and held there for 20 min. Maximal reaction rates and extent of assembly were determined for each sample and compared to a control reaction mixture in the same reaction medium containing 50 μM CO2, RPMI-1640 medium supplemented with 10% fetal bovine serum and antibiotics.

Compounds 12a–s and 17t–v were initially tested in vitro for antiproliferative activity against the K562 (human chronic myelogenous leukemia) cell line. These cell lines were grown at 37 °C in a humidified atmosphere containing 5% CO2, RPMI-1640 medium supplemented with 10% fetal bovine serum and antibiotics.

K562 cells were suspended at a density of 1 × 106 cells/ml in growth medium, transferred to a 24-well plate (1 ml/well), cultured with or without (in the case of control wells) a screening concentration of 10 μM compounds and incubated at 37 °C for 48 h. Numbers of viable cells were determined by counting in a hemocytometer after dye exclusion with trypan blue [17]. The antiproliferative effects of the compounds were estimated in terms of % growth inhibition, comparing cell viability of treated and untreated cells. We determined IC50 values (test agent concentration at half maximum test concentration) at which the cell proliferation was inhibited by 50% as compared with the untreated control for compounds that exhibited the best activity at the screening concentration.

4.2.2. Tumor cell line screening

The in vitro antiproliferative activity values were obtained by the Developmental Therapeutics Program, National Cancer Institute (USA) [18].

The human tumor cell lines of the cancer screening panel are grown in RPMI 1640 medium containing 5% fetal bovine serum and 2 mM l-glutamine. For a typical screening experiment, cells are inoculated into 96 well microtiter plates in 100 μl/well, cultured with or without (in the case of control wells) a screening concentration of 10 μM compounds and incubated at 37 °C for 48 h. Numbers of viable cells were determined by counting in a hemocytometer after dye exclusion with trypan blue [17]. The antiproliferative effects of the compounds were estimated in terms of % growth inhibition, comparing cell viability of treated and untreated cells. We determined IC50 values (test agent concentration at which the cell proliferation was inhibited by 50% as compared with the untreated control) for compounds that exhibited the best activity at the screening concentration.

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concentrations. Following drug addition, the plates are incubated for an additional 48 h at 37 °C, 5% CO₂, 95% air, and 100% relative humidity. For adherent cells, the assay is terminated by the addition of cold TCA. Cells are fixed in situ by the gentle addition of 50 μl of cold 50% (w/v) TCA (final concentration, 16% TCA) and incubated for 60 min at 4 °C. The supernatant is discarded, and the plates are washed five times with tap water and air dried. Sulforhodamine B (SRB) solution (100 μl) at 0.4% (w/v) in 1% acetic acid is added to each well, and plates are incubated for 10 min at room temperature. After staining, unbound dye is removed by washing subsequently solubilized with 10 mM trizma base, and the absorbance is read on an automated plate reader at a wavelength of 515 nm. For suspension cells, the methodology is the same except that the assay is terminated by fixing settled cells at the bottom of the wells by gently adding 50 μl of 80% TCA (final concentration, 16% TCA). Using the seven absorbance measurements [time zero (Tz), control growth (C), and test growth in the presence of drug at the five concentration levels (Tz)], the percentage growth is calculated at each of the drug concentration levels. Percentage growth inhibition is calculated as:

\[\frac{[Ti - Tz]}{C - Tz}] \times 100\text{ for concentrations for which } Ti \geq Tz\]

\[\frac{[Ti - Tz]}{Tz}] \times 100\text{ for concentrations for which } Ti < Tz\]

Three dose response parameters are calculated for each experimental agent. Growth inhibition of 50% (GI₅₀) is calculated from \([\frac{[Ti - Tz]}{(C - Tz)]} \times 100 = 50\), which is the drug concentration resulting in a 50% reduction in the net protein increase (as measured by SRB staining) in control cells during the drug incubation. The drug concentration resulting in total growth inhibition (TGI) is calculated from \(Ti = Tz\). The LC₅₀ (concentration of drug resulting in a 50% reduction in the measured protein at the end of the drug treatment as compared to that at the beginning) indicating a net loss of cells following treatment is calculated from \([\frac{[Ti - Tz]}{Tz}] \times 100 = -50\). Values are calculated for each of these three parameters if the level of activity is reached; however, if the effect is not reached or is exceeded, the value for that parameter is expressed as greater or less than the maximum or minimum concentration tested.

4.2.3. Cytotoxicity assays

To evaluate the number of live and dead neoplastic cells, the cells were stained with trypsin blue and counted on a hemocytometer. To determine the growth inhibitory activity of the drugs tested, 2 × 10⁵ cells were plated into 25 mm wells (Costar, Cambridge, UK) in 1 ml of complete medium and treated with different concentrations of each drug. After 48 h of incubation, the number of viable cells was determined and expressed as percent of control proliferation.

4.2.4. Morphological evaluation of apoptosis

Drug induced apoptosis was determined morphologically after labeling with acridine orange and ethidium bromide. Cells (2 × 10⁵) were centrifuged (300 × g), and the pellet was resuspended in 25 μl of the dye mixture. Ten μl of the mixture was examined under oil immersion with a 100× objective using a fluorescence microscope. Live cells were determined by the uptake of acridine orange (green fluorescence) and exclusion of ethidium bromide (red fluorescence) stain. Live and dead apoptotic cells were identified by perinuclear condensation of chromatin stained by acridine orange (100 μg/ml) or ethidium bromide (100 μg/ml), respectively, and by the formation of apoptotic bodies. The percentage of apoptotic cells was determined after counting at least 300 cells.

4.2.5. Determination of apoptosis by annexin-V

Cells (1 × 10⁶) were washed with phosphate-buffered saline (PBS) and centrifuged at 200 × g for 5 min. Cell pellets were suspended in 100 μl of staining solution containing FITC-conjugated annexin-V and propidium iodide (Annexine-V-Fluos Staining Kit, Roche Molecular Biochemicals, Mannheim, Germany) and incubated for 15 min at 20 °C. Annexin-V positive cells were evaluated by flow cytometry (Becton—Dickinson).

4.2.6. Flow cytometric analysis of cell cycle and apoptosis

Cells were washed once in ice-cold PBS and resuspended at 1 × 10⁶ ml in a hypotonic fluorescein solution containing propidium iodide (Sigma) 50 μg/ml in 0.1% sodium citrate plus 0.03% (v/v) nonidet P-40 (Sigma). After 30 min of incubation, the fluorescence of each sample was analyzed as a single-parameter frequency histogram using a FACScan flow cytometer (Becton—Dickinson, San Jose, CA). Distribution of cells in the cell cycle was determined using the ModFit LT program (Verity Software House, Inc.). Apoptosis was determined by evaluating the percentage of hypodiploid nuclei accumulated in the sub-G₀–G₁ peak after labeling with propidium iodide.

4.2.7. Immunofluorescence

Pt2K cells (Potoros tridactylis kidney epithelial cells) were obtained from the American Type Tissue Collection and grown as recommended by the supplier. The technique was described in detail previously [19,20]. Cells were treated with compounds for 24 h at 37 °C prior to fixation and staining with antibodies (Cy3 conjugate of anti-β-tubulin clone TUB2.1 and FTIC conjugate of anti-β-actin clone Ac-15 monoclonal antibodies from Sigma). Cells were examined with a Nikon Eclipse E800 microscope equipped with epifluorescence and appropriate filters, and images were collected with a Spot digital camera.

4.2.8. Tubulin assembly

The procedure with purified bovine brain tubulin was described in detail previously [21].

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References

[18] The methodology can be found at http://dtp.nci.nih.gov/.