Chem Biol Drug Des 2012

Research Article

doi: 10.1111/cbdd.12004

Evaluation of Perylenediimide Derivatives for Potential Therapeutic Benefits on Cancer Chemotherapy

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Perylene derivatives, known to have potential therapeutic benefits on particular cancer types as photosensitizers, may also function as small-molecule inhibitors with promising therapeutic value for diverse diseases. This recently recognized biological activity was attributed to their capacity to modulate the function of various enzymes as biological targets *in vitro*. Although the inhibitory activity on glutathione transferase and Src tyrosine kinase is important in determining the anticancer potential of compounds for target-specific drug design and development, to date, there are no successful inhibitors of this kind. Moreover, there are only a few studies about the effects of perylene derivatives on glutathione transferase and various kinases. In this study, four novel perylene compounds, *N,N*-disubstituted perylenediimides and their 1,7-dibromo derivatives, were synthesized and evaluated for their biological activities. Here, among the compounds analyzed, one of them was identified with strong glutathione transferase inhibition and two with dual activity for both glutathione transferase and *c-src* inhibition. These results revealed that perylene derivatives may be employed as potential chemosensitizers to prevent chemotherapy-dependent drug resistance and identified as prospective anticancer agents with dual activity on both glutathione transferase and *c-src* enzymes.

Key words: anticancer agent, glutathione transferase, perylenediimide, small-molecule inhibitor, tyrosine kinase

Received 22 January 2012, revised 12 June 2012 and accepted for publication 16 July 2012

Perylene derivatives are compounds used in a variety of industrial applications for decades, especially in the dye-sensitized solar cells, organic light-emitting diodes, semiconductors and organic thin film transistors, as well as in chemical oxidations as photosensitizers (1–3). The most commonly analyzed perylene derivatives were from hypericin (1,3,4,6,8,13-hexahydroxy-10,11-dimethylphenanthro[1,10,9,8-*opqra*]perylene-7,14-dione), which is the most potent, naturally occurring pro-oxidant that facilitate, or induce, the apoptosis and necrosis in a wide spectrum of cancer cell types (4). Moreover, these compounds have shown some antiviral, antidepressant, and antibacterial activities, and still in use as part of natural food supplements available in the markets (4–6). The current use of hypericin and other perylene derivatives in biomedical applications is usually based on their capacity to produce free radicals or reactive oxygen species (ROS) upon radiation, so-called photodynamic therapy (7). Here, the nontoxic compound, administered systemically, locally, or topically, acts as photosensitizer and during the tissue irradiation becomes active and generate ROS. Eventually, the increased ROS levels cause cytotoxicity on tumor cells leading to death and tissue destruction (8).The toxicity of non-radiated photosensitizer is a critical issue for these applications, because the administered compound directly interacts with metabolic detoxification system enzymes, and upon irradiation, the resulting product may become less effective or more toxic to the organism. As a defense mechanism, the detoxification system enzymes are evolved and can be induced to protect the organism against toxic effects of chemicals that may be harmful to their survival (9). Among these enzymes, mammalian glutathione transferase (GST, EC 2.5.1.18) is one of the most important detoxification enzymes that catalyze the nucleophilic addition of glutathione to diverse electrophilic molecules (10) and solubilize them to facilitate the transport of toxic substances from cells. On the other hand, GSTs have emerged as promising therapeutic targets because the specific isozymes are either overexpressed, or overactivated, in a wide variety of diseases, including cancer. Furthermore, current evidence showed the role of GSTs in signal transduction events is fundamental for cellular functioning (10–12) through the protein tyrosine kinase (PTK)–mediated GST activation and signaling from cell membrane to nucleus (13–17).Signal transduction is under tight control, provided by signaling components, such as kinases, and also by detoxification system enzymes, which are mostly the GSTs. For therapeutic purposes, the use of small molecules interfering with signaling components was shown to induce drug resistance as a result of GST-mediated detoxification reactions or signaling events. Therefore, in this context, a series of perylenediimides has been evaluated for their ability to bind DNA and their potential to induce cytotoxic effects on multiple

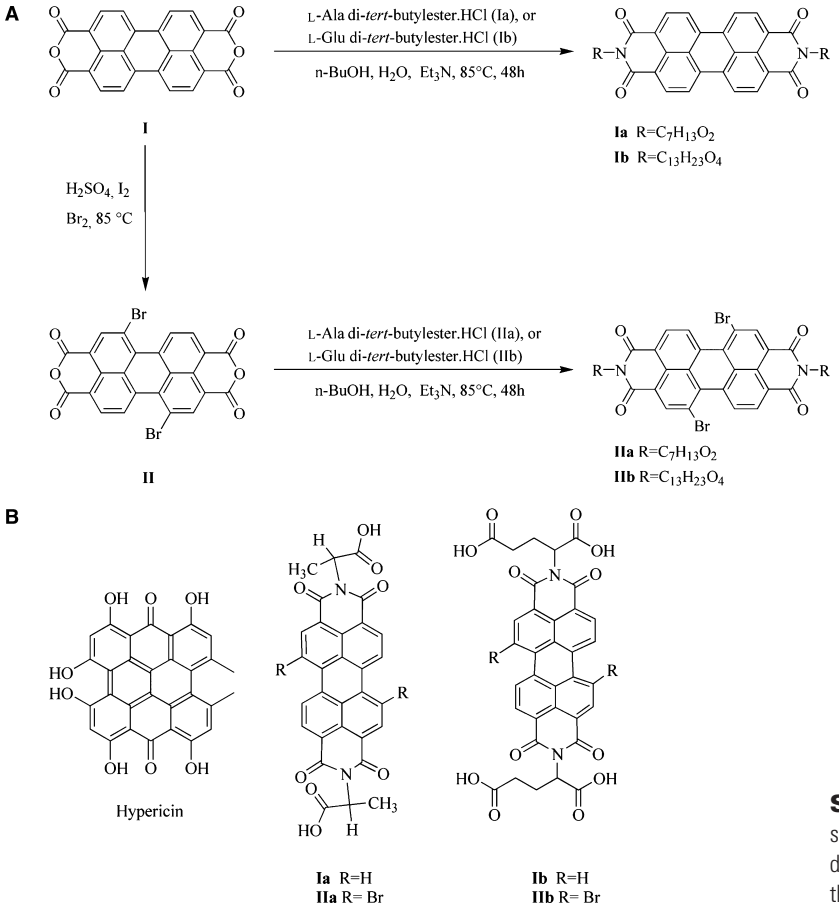
cancer cell lines (18–22). However, such studies, searching the biological effects, were focused mainly on cytotoxic effects of perylene derivatives on cell viability, without mentioning the signaling components that contribute to the therapeutic effectiveness of compounds. In signal transduction from extracellular stimuli through cell membrane to cytoplasm, and to the nucleus, Src kinase (Src), among the PTKs, emerges as a fundamental constituent with essential roles in diverse signaling mechanisms (23). Therefore, the deregulations in its activity have been associated with various pathologic conditions, including cancer. In this context, studies with hypericin in photodynamic therapy applications revealed its association with GST and various members of PTK, where the enhanced photodynamic response was reported with GST inhibition at low nanomolar ranges of compound administered, and related with the decreased GST-dependent resistance (19–22).

Perylenediimides are known for their photo- and chemical stability. However, they do not have absorptions in the red end of the visible spectrum to be suitable for photodynamic applications. Our previous efforts to design and develop novel perylenediimide derivatives resulted in compounds with potential efficacy in photodynamic therapy (24–26). Recently, green perylenediimides with dialkylamino substituents on the perylene core was reported as an alternative photosensitizer for photodynamic therapy (27). Previously, we synthesized water-soluble green perylenediimide dyes with absorption peaks beyond 650 nm as an alternative photosensitizer for photodynamic therapy (24). With these compounds, we demonstrated that the cytotoxicity of derivatives on red-light excitation was more significant compared to non-excited compounds. These preliminary *in vitro* experiments, hence, verified their potential utility in photodynamic therapy. Consistent with these efforts, in this current study, we report the design and synthesis of novel perylenediimides (Scheme 1: panel A) with amino acid substitutions (Scheme 1: panel B) and their inhibitory characteristics against two critical enzyme targets. To reveal their potential to minimize tissue resistance while improving anticancer effectiveness, the structure-activity relationships were assessed by dose–response studies.

Experimental Section

Chemistry

L-glutamic acid di-tert-butylester hydrochloride, L-alanine tert-butylester hydrochloride, n-butanol, and the tyrosine kinase inhibitors Genistein and SU6656 were purchased from Sigma-Aldrich, St. Louis, MO, USA. 3,4:9,10-perylenetetracarboxylic acid 3,4:9,10-dianhydride, sulfuric acid, triethylamine, acetic acid, trifluoroacetic acid, liquid Br_2, silica gel 60 (particle size: 0.040–0.063 mm, 230–400 mesh ASTM), silica gel 60 Kiesel gel F_{254} TLC Aluminum Sheets were purchased from Merck, Darmstadt, Germany. Takara Universal



Scheme 1: Panel A- the synthesis of *N,N'*-disubstitutedperylenediimides and their 1,7-dibromo derivatives. Panel B- structures of hypericin and the synthesized novel erylenediimide derivatives.

Tyrosine Assay ELISA plates and reagents were purchased from Takara-Bio Inc., Shiga, Japan; recombinant Src and Profluor Fluorescent Tyrosine Kinase reagents were purchased from Promega, Madison, WI, USA. Bovine liver cytosolic fraction was used as GST enzyme source and prepared in our laboratory. Other chemicals were at analytical grade and purchased from Sigma-Aldrich. The nuclear magnetic resonance spectra (^{1}H- and ^{13}C-NMR) were recorded using Avance Series-DX-400 (δ given in ppm, J in Hz), Bruker Instruments, Madison, WI, USA. All chemical shift values were expressed in parts per million (ppm) relative to tetramethylsilane (Me_4Si) as an internal standard, and signals were reported as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet). Electrospray ionization (ESI) mass spectra were recorded on Agilent 6500 Series LC-MS spectrometer, Agilent instruments, Paolo Alto, CA, USA. The kinetic and end-point detection to measure the substrate phosphorylation or glutathione (GSH) conjugation was performed with absorbance- and fluorescence-based assays and recorded by SpectraMax-M2e Multi-Mode Microplate Reader, Molecular Devices Corporation, Sunnyvale, CA, USA.

Synthesis of 1,7-dibromo-3,6:9,10-perylenetetracarboxylic acid dianhydride (II)

A mixture of 1.47 g (3.74×10^{-3} mol) perylene-3,4:9,10-tetracarboxylic acid dianhydride (**I**), 12.01 mL H_2SO_4 and 0.032 g (1.26×10^{-4} mol) I_2 was stirred for 12 h at room temperature (RT) and heated to 85 °C for 30 min. After cooling to RT, 2.1 mL Br_2 was added to the mixture, in the pressure tube, within 8 h. The mixture was heated to 85 °C, and then, 3 mL Br_2 was added for a period of 12 h. The concentration of H_2SO_4 was decreased to 86% by adding 1.67 mL of water in 1 h. Finally, the mixture was cooled to RT, filtered under suction through a G-4 glass-frit filtering crucible, washed with 15 mL H_2SO_4 (86% w/w), and then with H_2O. The bright-red precipitate was dried in a vacuum oven at 120 °C for 24 h (yield, 82%).

$C_{24}H_6Br_2O_6$.

Synthesis of *N,N'*-(L-alanine tert-butylester)-3,4:9,10-perylenediimide (I-a)

0.5 g (1.274×10^{-3} mol) perylene-3,4:9,10-tetracarboxylic acid dianhydride and 0.462 g (2.548×10^{-3} mol) alanine tert-butylester hydrochloride were dissolved in 10 mL H_2O, 10 mL n-butanol and 1.5 mL triethylamine and stirred for 48 h at 85 °C. After solvent removed, the product was purified via column chromatography (trichloromethane/methanol, 97:3), and dried *in vacuo* (yield, 32%).

$C_{36}H_{30}N_2O_8$, ESI-MS: m/z 646.22 ($M^+ + 1$).

^{1}H NMR (400 MHz, $CDCl_3$), δ[ppm]: δ 1.52 (s, 18H, -CH_3), 1.71 (d, J = 7 Hz, -CH_3), 5.68 (m, 2H, N-CH), 8.35 (d, J = 8.08 Hz, 4H, CH-arom), 8.49 (d, J = 7.96 Hz, 4H, CH-arom).

^{13}C NMR (100 MHz, $CDCl_3$), δ[ppm]: 14.79 (aliphatic), 27.96, 29.60, 50.15, 81.47, 122.72, 123.09, 125.99, 129.10, 131.20, 134.17 (aromatic), 162.15 (1-amide), 168.57 (C=O).

Synthesis of *N,N'*-(L-glutamic acid tert-butylester)-3,4:9,10-perylenediimide (I-b)

0.5 g (1.274×10^{-3} mol) perylene-3,4:9,10-tetracarboxylic acid dianhydride and 0.753 g (2.548×10^{-3} mol) glutamic acid di-tert-butylester hydrochloride were dissolved in 10 mL H_2O, 10 mL n-butanol and 1.5 mL triethylamine and stirred for 48 h at 85 °C. After solvent removed, the product was purified via column chromatography (trichloromethane/methanol, 97:3) and dried *in vacuo* (yield, 35%).

$C_{50}H_{54}N_2O_{12}$, ESI-MS: m/z 874.36 ($M^+ + 1$).

^{1}H NMR (400 MHz, $CDCl_3$), δ[ppm]: 1.39 (s, 36H, -CH_3), 2.36 (m, 4H, -CH_2), 2.65 (m, 4H, -CH_2), 5.65 (t, 2H, N-CH), 8.45 (d, J = 8.12 Hz, 4H, CH-arom), 8.55 (d, J = 9.6 Hz, 4H, CH-arom).

^{13}C NMR (100 MHz, $CDCl_3$), δ[ppm]: 24.13, 27.97, 32.41, 53.83, 76.80, 81.71 (aliphatic), 122.96, 126.22, 129.36, 131.45, 134.41 (aromatic); 162.59 (1-amide); 167.97; 171.49 (C=O).

Synthesis of 1,7-dibromo-*N,N'*-(L-alanine tert-butylester)-3,4:9,10-perylenediimide (II-a)

0.5 g (9.089×10^{-4} mol) 1,7-dibromo-perylenetetracarboxylic acid 3,4:9,10 dianhydride (**II**) and 0.33 g (1.817×10^{-3} mol) alanine tert-butylester hydrochloride were dissolved in 10 mL H_2O, 10 mL n-butanoland 1.5 mL triethylamine and stirred for 48 h at 85 °C. After solvent removed, the product was purified via column chromatography (trichloromethane/methanol, 98:2), and dried *in vacuo* (yield, 30%).

$C_{36}H_{28}Br_2N_2O_8$, ESI-MS: m/z 804.049 ($M^+ + 1$).

^{1}H NMR (400 MHz, $CDCl_3$), δ[ppm]: 1.49 (s, 18H, -CH_3), 1.67 (d, J = 7.09 Hz, -CH_3), 5.22 (m, 2H, N-CH), 8.71 (d, J = 8.161 Hz, 4H, CH-arom), 8.92 (s, 2H, CH-arom), 9.5 (d, J = 8.16 Hz, 4H, CH-arom).

^{13}C NMR (100 MHz, $CDCl_3$), δ[ppm]: 24.06, 29.74, 60.74, 81.11 (aliphatic), 117.82, 123.18, 128.43, 132.84 (aromatic), 168.30 (1-amide), 184.64 (C=O).

Synthesis of 1,7-dibromo-*N,N'*-(L-glutamicacid tert -butylester)-3,4:9,10-perylenediimide (II-b)

0.5 g (9.089×10^{-4} mol) 1,7-dibromo-perylenetetracarboxylic acid 3,4:9,10 dianhydride (**II**) and 0.537 g (1.817×10^{-3} mol) alanine tert-butylester hydrochloride were dissolved in 10 mL H_2O, 10 mL n-butanol and 1.5 mL triethylamine and stirred for 48 h at 85 °C. After solvent removed, the product was purified via column chromatography (trichloromethane/methanol, 95:5) and dried *in vacuo* (yield, 32%).

$C_{50}H_{52}Br_2N_2O_{12}$, ESI-MS: m/z 1032.18.

^{1}H NMR (400 MHz, $CDCl_3$), δ[ppm]: 1.36 (s, 36H, -CH_3), 2.3 (m, 2H, -CH_2), 2.7 (m, 2H, -CH_2), 5.6 (t, 2H, N-CH), 8.7 (d, J = 8.16 Hz, 2H, CH-arom), 8.9 (s, 2H, CH-arom), 9.5 (d, J = 8.16 Hz, 2H, CH-arom).

^{13}C NMR (100 MHz, $CDCl_3$), δ[ppm]: 29.26, 30.18, 32.31, 45.30, 80.51 (aliphatic), 127.45, 130.18, 137.04, 138.12, 144.26 (aromatic), 167.59 (1-amide), 168.73, 171.38 (C=O).

Evaluation of perylene derivatives through glutathione transferase assays

The slightly modified method of Habig (28) was used to measure the kinetic change in substrate (GSH) utilization by GST. Then, the method was adopted for microscale applications. Briefly, the total GST activity was measured using reaction buffer (100 mM potassium phosphate buffer at pH 6.5 containing 2.4 mM CDNB and 3.2 mM GSH) (29,30). In the assay, the compounds were incubated with reaction buffer at RT (22–25 °C) for 5 min. Upon addition of enzyme (bovine liver cytosol), the plate was mixed for 30 seconds, and GSH-CDNB conjugate formation, as an increase in the absorbance, was monitored at 340 nm for 240 seconds. Initial rates of enzymatic reactions were determined for the formation of GSH-conjugation product, and the activity of enzyme samples was determined as nmol/minute/mg protein, where the total protein content was determined by the Lowry method. For the analysis of compounds, the kinetic measurements were performed with compounds dissolved in dimethyl sulfoxide (DMSO, 0.5% per assay volume), and the calculations were made with respect to vehicle control (the assay buffer with 0.5% DMSO) without compounds. The maximum inhibition was determined using 100 mM of ethacrynic acid as positive control. The dose–response curves were constructed using the absorbance change (enzyme activity in the assay) from the kinetic data and reported as OD_{340}/min. All measurements were performed in 96-well microplate, using Spectramax M2e. IC_{50} values were determined as the concentration of a compound required to achieve 50% inhibition of enzyme activity with respect to the vehicle control and determined by nonlinear regression analysis, the four parameter logistic equation (Sigmoidal dose–response, GraphPad Prism version 4.0 for Windows, GraphPad Software, San Diego, CA, USA).

Evaluation of perylene derivatives through in vitro tyrosine kinase assays

The inhibition of the synthesized compounds was determined by virtue of the ELISA based *in vitro* Tyrosine Kinase Assay (Takara Universal Protein Tyrosine Kinase Assay, Takara Corp., Tokyo, Japan) and fluorescent dye Rhodamine 110 (R110) based indirect phosphotyrosine phosphatase based kinase assay (Profluor; Promega Inc., Madison, WI, USA). The kinase assay was performed at 37 °C, with 40 nM ATP (final assay volume of 50 μL). The enzyme activity was probed with HRP-conjugated anti-phosphotyrosine (PY20) antibody at 450 nm (31). The ELISA assay was used to determine the 80% active Src concentration for fluorescent end-point detection of kinase activity. The activities of the Src to construct the calibration curve were as follows: 1520, 760, 380, 190, 94.8, and 47.4×10^{-7}, units/μL. Then, the inhibitory activities of compounds were monitored by fluorescent end-point detection, that is, ProFluor Assay Protocol, with some modifications (31). In this assay, the pure enzyme Src (Promega) was used to achieve 20% of the maximum fluorescent signal (80% active enzyme) and the compounds were prepared at final concentrations of 4.50 mM to 0.10 μM in DMSO. Briefly, the molecules were mixed with Src-family kinase R110 substrate, and the reaction was initiated with the addition of ATP, followed by incubation at 22 °C for 60 min. The protease solution was added, and mixture was incubated another 60 min at RT. The reaction was terminated with protease inhibitor, and the fluorescence of R110 was read at 525 nm (Ex 460 nm). The decrease in fluorescence of each microplate well is inversely related to the kinase activity of the enzyme within the wells and comparison performed with respect to control wells. This assay also monitors the AMC fluorescent signal, which is the coumarin derivative without phosphorylation site available, to evaluate whether the observed kinase inhibition results are false positive or not. The IC_{50} values of compounds were determined by comparing the Src activity of the wells containing compounds, with the activities of vehicle and positive control (genistein and SU6656) wells.

Results and Discussion

The drawbacks in cancer research mainly because of the cellular resistance to chemotherapeutics and the toxic side-effects of drugs. Therefore, to develop therapeutics with full biological response, it appears critical to evaluate their interactions with drug metabolizing enzymes, such as GSTs, and their possible contribution to PTK-mediated signaling mechanisms, such as Src. Depending on duration, dosage, and chemical composition of chemotherapeutics administered, the acquired resistance is commonly experienced problem and associated with the overactive GST isozymes. In an attempt to overcome this difficulty, in particular linked to systemic toxicity, various compounds that possibly modify the activities of detoxification system enzymes have been explored. For this purpose, we described the synthesis and chemical characterization of 3,6:9,10-perylenediimide derivatives as new compounds to be developed for selective transferase and kinase inhibitors. The required dibromo-substituted perylenediimide **II** was synthesized similar to the practical and straightforward synthesis with perylene-3,4:9,10-tetracarboxylic acid dianhydride **I** as a starting material (26). Amino acid modifications on the imide position of perylenedianhydride were considered to improve the compound solubility in physiological medium and hence the improved absorption by cells. In this context, the most circulating amino acids of human metabolism, namely alanine and glutamic acid, were chosen for substitution. The reaction of tetracarboxylic acid anhydride with either aliphatic or aromatic primary amines was shown that the corresponding diimides were in good yield. Similarly, tert-butylester derivative of amino acid with **I** or **II** afforded perylenediimides with more than 30% yield. We have synthesized four novel perylenediimides (Scheme 1: panel A): *N,N′*-(L-alanine tert-butylester)-3,4:9,10-perylenediimide (I-a), *N,N′*-(L-glutamic acid tert-butylester)-3,4:9,10-perylenediimide (I-b), 1,7-dibromo-*N,N′*-(L-alanine tert-butylester)-3,4:9,10-perylenediimide (**Ia**), and 1,7-dibromo-*N,N′*-(L-glutamic acid tert-butylester)-3,4:9,10-perylenediimide (**Ib**), where tert-butyl ester of L-alanine and di-tert-butyl ester of L-glutamic acid were used as amine substituents. The chemical characterization of compounds was verified by ^{1}H- and ^{13}C-NMR and MS (ESI) analysis, and the spectral properties were investigated in chloroform. The tert-butylester groups of amino acids were removed before biological assays to improve the solubility of compounds in aqueous buffers because of the nature of amino acid substituents and in common organic solvents because of the perylene core. The tert-butyl protection on propionic (alaninyl) and pentanedioic acid (glutamyl) functions on N atom were removed by trifluoroacetic-acid/chloroform treatment (Scheme 1, panel A,B). Then, the compounds (**Ia**–**IIb**) were evaluated for their inhibitory activities toward tyrosine phosphorylation of Src and S-glutathionylation of

GST. Similar to our previous approach, we also investigated the effects of substituents at bay position that is the region located perylene core scaffold and the amino acid substitution to form piperidine from pyran ring. The core compounds **I** and **II** did not show any inhibition profile, where the IC_{50} values were not assessable. Compounds **IIa** and **IIb** turned out to be strong inhibitors of GST with IC_{50} values of 106.3 ± 27.3 (Figure 1) and 709.4 ± 23.6 nM (Figure 2), respectively, and are more effective than compound **Ia** with IC_{50} of 2.044 ± 0.032 μM (Figure 3). These important differences in the inhibitory potencies and profiles were possibly due to the presence of bromide in the bay region of perylene core and considered to increase the GST affinity toward these molecules. Notably, **Ia** (Figure 4) and **IIb** (Figure 5) showed inhibition of Src with IC_{50} values of 433.1 ± 11.9 mM and 632.3 ± 66.8 μM, respectively. The compounds **Ib** and **IIa** showed very low inhibition against Src (IC_{50} > 1 mM), where the dose–response curves did not fit 4-parameter logistic equation (data not shown). Neither changes in the final concentrations in assay, nor the alterations in reaction conditions resulted in reasonable effect of **Ib** or **IIa** on target enzyme activities. This is the first demonstration of a possible anticancer activity of the novel perylenediimide derivatives with amino acid functional groups, without irradiation, and the first report in investigating their effects on Src, and cytosolic GSTs. As the GSTs are highly overexpressed in cancer cells, their inhibition by perylenediimides might offer new clues on their mechanism of action and open interesting perspectives for future tumor therapies.

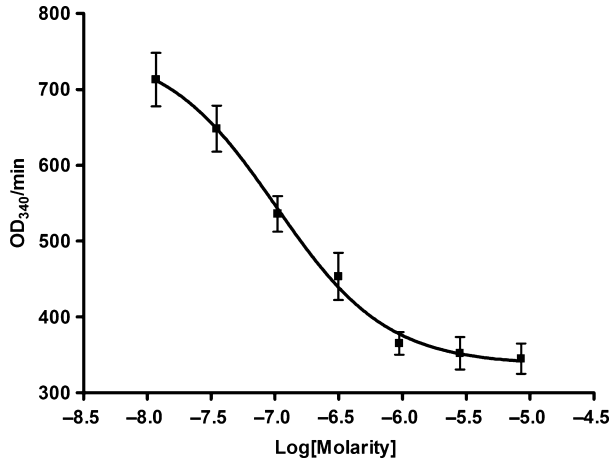


Figure 1: Dose–response curve for glutathione transferase enzyme activity (OD_{340}/min) against varying doses of compound **IIa**, where the IC50 value is 106.3 ± 27.3 nM.

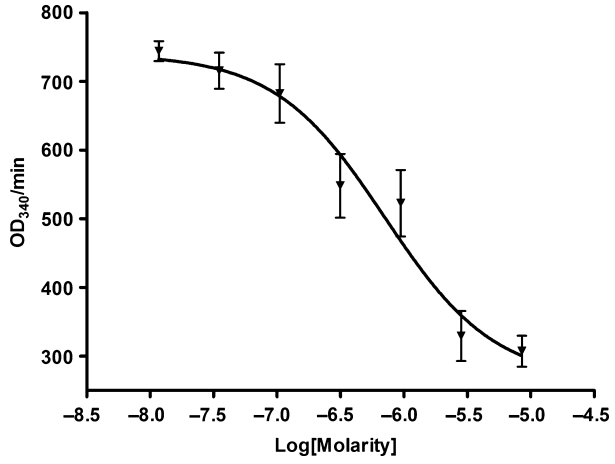


Figure 2: Dose–response curve for glutathione transferase enzyme activity (OD_{340}/min) against varying doses of compound **IIb**, where the IC50 value is 709.4 ± 23.6 nM.

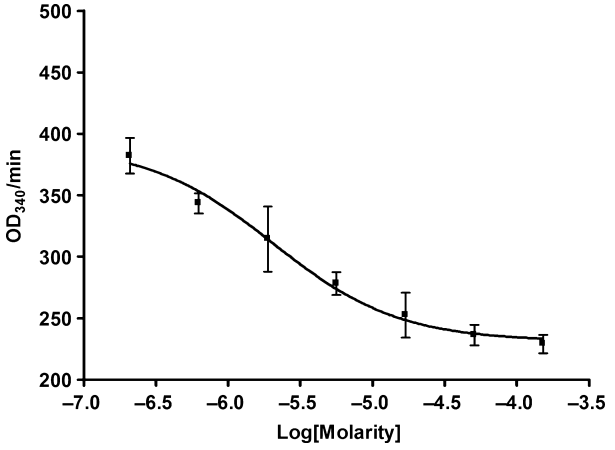


Figure 3: Dose–response curve for glutathione transferase enzyme activity (OD_{340}/min) against varying doses of compound **Ia**, where the IC50 value is 2.044 ± 0.032 μM.

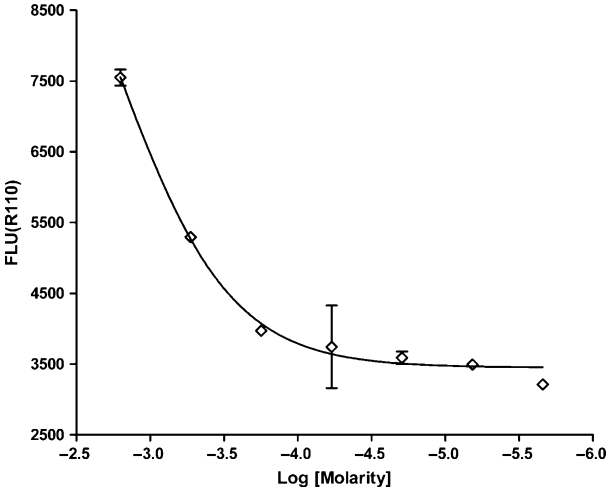


Figure 4: Dose–response curve for Src enzyme activity presented as change in fluorescence against varying doses of compound **Ia**, where the IC50 is 433.1 ± 11.9 μM.

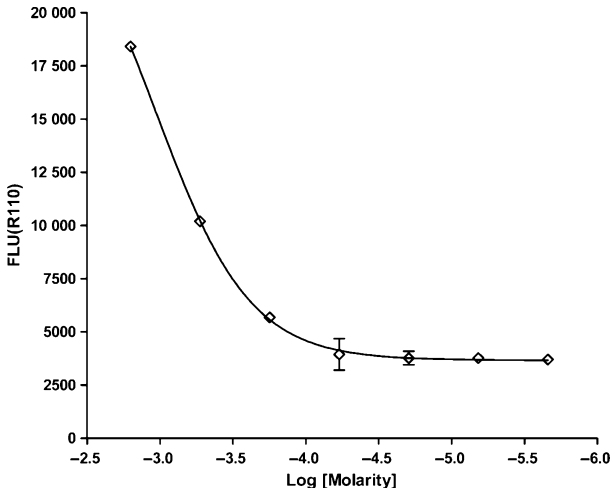


Figure 5: Dose–response curve for Src enzyme activity presented as change in fluorescence against varying doses of compound **IIb**, where the IC50 is 632.3 ± 66.8 μM.

Acknowledgments

This work was partially supported by grants from the Atilim University (BAP 2011), Balikesir University (BAP2009/40; BAP 2010/18), The Scientific and Technological Research Council of Turkey (TUBITAK-110T026), and Undersecretariat of State Planning Organization (DPT-2005-K-120-170).

Conflict of Interest

The authors have declared no conflict of interest.

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