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Original article

# Synthesis of novel aminoquinonoid analogues of diospyrin and evaluation of their inhibitory activity against murine and human cancer cells

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**Abstract**

The synthesis and tumor-inhibitory activity of a series of aminonaphthoquinone derivatives of diospyrin, which was isolated from *Diospyros montana* Roxb., are presented here for the first time. An aminoacetate derivative showed the maximum (~93%) increase in life span in vivo against murine Ehrlich ascites carcinoma (EAC) at a dose of 1 mg $kg^{-1}$ $day^{-1}$ (ip; five doses), and the lowest $IC_{50}$ (0.06 μM) in vitro. Further, the same analogue also exhibited considerable enhancement in antiproliferative activity when evaluated against human cell lines, viz. malignant skin melanoma and epidermoid laryngeal carcinoma ($IC_{50}$ = 0.06 and 0.92 μM, respectively) in comparison to the natural precursor, diospyrin ($IC_{50}$ = 0.82 and 3.58 μM, respectively). Moreover, diospyrin and all its derivatives were found to show significantly greater (~17- to 1441-fold) cytotoxicity against the tumor cells as compared to normal human lymphocytes. All these quinonoids generated substantial amounts of reactive oxygen species in EAC cells, more or less commensurate to their respective $IC_{50}$ values.


*Keywords:* Diospyrin; Aminonaphthoquinonoids; Antitumor activity; Ehrlich ascites carcinoma; Malignant skin melanoma; Epidermoid laryngeal carcinoma

## 1. Introduction

Quinonoids occur widely in animals, plants and microorganisms, and often carry out indispensable roles in the biochemistry of energy production by providing vital links in the respiratory chain of living cells. These compounds act as inhibitors of electron transport, uncouplers of oxidative phosphorylation, and give rise to a wide range of cytostatic and antiproliferative activities [1]. Hence, quinonoids, such as doxorubicin, salvicine, saframycin, saintopin, actinomycin D, etc., have been widely used in cancer chemotherapy [2]. Incidentally, the molecular framework of several such clinical agents contains the aminoquinonoid moiety as the key structural component [3], e.g., mitomycin C, mitoxantrone, streptonigrin, etc., which were obtained either from natural sources or through chemical synthesis. Thus, in order to find novel 'lead molecules', continuous efforts are under way to procure structurally diverse quinonoids possessing amino group(s), − either free or substituted, often forming part of a heterocyclic ring [4–9]. Some of these aminoquinonoids have exhibited marked cytotoxicity towards human cancer cell lines [8,10–12].

In our laboratory, diospyrin (**1**), a plant-derived bisnaphthoquinonoid compound, was observed to inhibit the growth of murine tumor in vivo [13]. Recently, significant enhancement of this activity was achieved through liposomal encapsulation of **1** [14]. Further, its synthetic derivatives were found to

*Abbreviations:* DCF, 2′,7′-dichlorofluorescein; DCFH, 2′,7′-dichlorodihydrofluorescein; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; DMEM, Dulbecco's modified Eagle's medium; EAC, Ehrlich ascites carcinoma; FCS, fetal calf serum; $IC_{50}$, inhibitory concentration at 50% cell death; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; NAC, *N*-acetyl-L-cysteine; PBMC, peripheral blood mononuclear cells; R.F.I., relative fluorescence intensity; ROS, reactive oxygen species; RPHPLC, reversed phase high performance liquid chromatography.

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exhibit improved anticancer activity and induce apoptosis in cancer cell lines [15–19]. Thus, **1** was envisaged as a potential 'lead molecule' for development of new therapeutic strategy against cancer. Here, novel amino-substituted analogues of **1** have been synthesized and their antiproliferative activity evaluated for the first time in human malignant skin melanoma (A375) and epidermoid laryngeal carcinoma (Hep2) cell lines, following their studies in Ehrlich ascites carcinoma (EAC) tumor model in Swiss A mice, in vitro as well as in vivo. Furthermore, cytotoxicity of all the quinonoid analogues was also assessed against normal human lymphocytes.

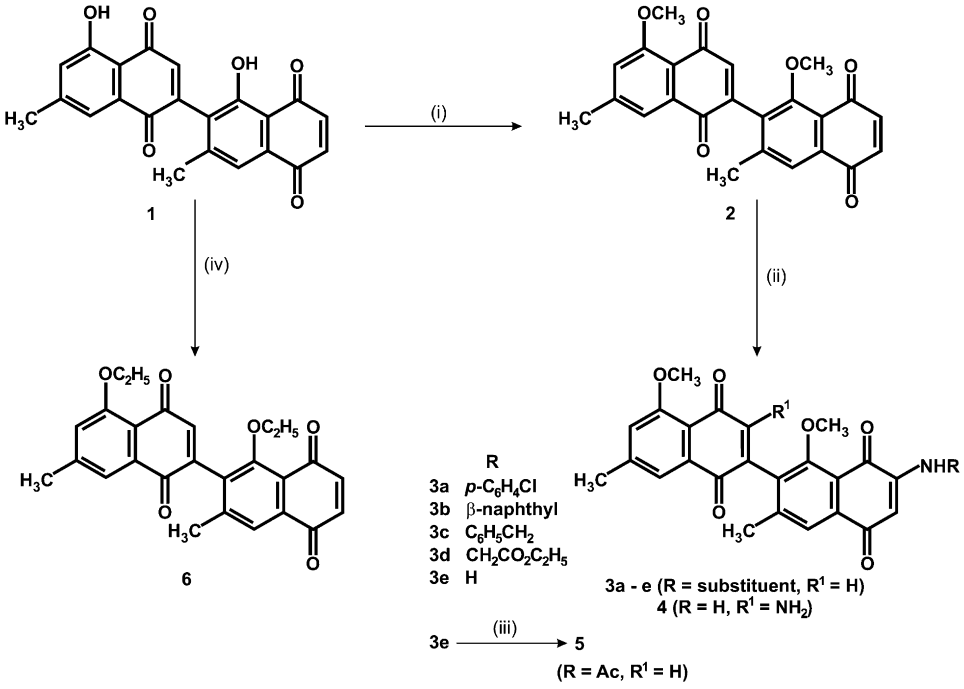
The wide spectrum of biological activity of many quinonoids including derivatives of diospyrin is related to their ability to generate reactive oxygen species (ROS) via a facile redox cycling mechanism [18,20]. Generally, the electron-accepting or electron-donating character of the substituents would determine the capacity of electron transfer in the quinonic system. However, introduction of vicinal oxidizable groups, such as amino substituents, in quinones would provide further modulation of the redox properties, inducing oxidative stress in the cells, and alkylation of cellular macromolecules, including lipids, proteins and DNA [21]. Recently, studies have been initiated to correlate the electrochemical behaviour of quinonoids with their biological activity [22]. Keeping in view the critical role of ROS and associated oxidative stress in the mechanism of apoptotic cell death in tumor cells, the capacity of the novel aminoquinonoids to generate ROS has been presently estimated by fluorimetric measurements, followed by cyclic voltammetric determination of the redox potential of the same compounds.

## 2. Results and discussion

### 2.1. Chemistry

#### 2.1.1. Synthesis

Diospyrin (**1**) was isolated [13,23] in a fairly large quantity (~5 g) from the stem bark (5 kg) of *Diospyros montana* Roxb. (family: Ebenaceae; authentic stem bark kindly provided by Dr. R.B. Mishra, Nagarjuna Education Society, Bolangir, Orissa). The structure has finally been confirmed to be 2,6′-bis(5-hydroxy-7-methyl-1,4-naphthoquinone) through total synthesis [24], and recently, by crystallographic analysis [25]. Using methyl iodide and silver oxide, **1** was converted to its dimethyl ether derivative (**2**) [23], which was used as a synthon to obtain the analogues with amino substituents, both aliphatic as well as aromatic (Scheme 1). The site-specific reaction condition for the conventional 1,4-Michael addition, followed by aerial oxidation, was optimized specifically for each of these derivatives. As expected, the reaction time and temperature required to prepare **3a** and **3b**, by nucleophilic addition of **2** with the two aromatic aminating agents, were found to be substantially greater than those required to prepare the aliphatic amines (1–3 h; ~0 °C). Thus, **2** on refluxing with *p*-chloroaniline at 80 °C for 10 h afforded **3a**, while **3b** was prepared by vigorously stirring **2** with β-naphthylamine at room temperature for 6 days. For converting **2** into its ethyl glycinate derivative (**3d**), the corresponding hydrochloride was taken as the aminating agent. In this reaction, 80% yield was achieved by using sodium acetate as a mild base, which was preferable over triethylamine producing




Scheme 1. Synthesis of amino derivatives of diospyrin. Reagents and conditions: (i) $CH_3I$, $Ag_2O$, $CHCl_3$, stir, rt, 6 h; (ii) $RNH_2$, $CHCl_3$, $C_2H_5OH$, 1 h to 6 days; (iii) acetyl chloride, pyridine, stir, rt, 30 min; (iv) $C_2H_5I$, $Ag_2O$, $CHCl_3$, stir, rt, 8 h.

only 22% of **3d**. However, triethylamine was quite appropriate for preparing the amino derivative (**3e**; 66%) from **2**, by stirring with hydroxylamine hydrochloride at ice-cold condition for 1 h. Here, it is to be noted that contrary to the expectation, use of hydroxylamine hydrochloride did not lead to an oxime [26]; neither was the 1,4-Michael adduct of the quinonoid substrate obtained. Incidentally, Bittner and Lempert had also carried out similar reactions and had suggested a mechanism involving 1,4-addition–aromatisation–reductive elimination [27]. Also, they had used several *O*-alkylhydroxylamines for this purpose and found that *O*-benzylhydroxylamine hydrochloride to be the best aminating agent. However, this reagent, when applied to our substrate **2**, afforded only the diamino product, **4** (37%), instead of the expected monoamine (**3e**), as the major one. Nevertheless, formation of benzyl alcohol in this reaction, as was confirmed by NMR analysis, did corroborate the aforesaid mechanism in our case also; apparently, the disparity in yield of the major product resulted due to a difference in reactivity of the dimeric substrates, in contrast to the monomeric ones used by Bittner and Lempert [27].

Structure of the prepared amino derivatives was characterized through standard instrumental techniques involving spectroscopic (UV, IR, NMR and MS) studies and elemental analyses. The 3′-position of the amino group in these derivatives was unequivocally confirmed by the correlation NMR spectroscopic studies through complete $^{1}H$ and $^{13}C$ NMR spectra [19].

### 2.1.2. Redox potential

Cyclic voltammetric measurement was carried out to determine the redox potential of the aminonaphthoquinonoids. All of them gave well-defined quasi-reversible waves, the cathodic peak relating to the reduction of quinone, and the anodic one due to its reoxidation. A drastic shift of the peak positions towards more negative potential was observed when **1** ($E_{1/2}$ −344 mV), with its free −OH groups at 5- and 5′-positions, was converted to its alkyl ether, **2** ($E_{1/2}$ −603 mV), followed by its amino analogues, **3a–e**, **4** and **5** ($E_{1/2}$ −707 to −919 mV; Table 1). The markedly higher $E_{1/2}$ value of **1** could be attributed to the electron-accepting inductive effect of its hydroxyl oxygen, together with the intramolecular hydrogen bonding, which would further stabilise the resultant anion through delocalisation. Hence, the absence of hydrogen bonding, reinforced by the electron-donating mesomeric effect of the methoxy groups, would cause the observed shift in $E_{1/2}$ value in **2** and its derivatives [28]. Again, the redox potentials of **3a–e**, **4** and **5** were significantly lower than those of **2** due to the additional inductive and delocalisation effects of the amino groups, which would enhance the electron density of the quinonic system and thereby make it more difficult to reduce.

Table 1
Redox potential of diospyrin analogues measured by cyclic voltammetry[a]

| Compound | $E_c$[b] (V) | $E_a$[c] (V) | $E_{1/2} = (E_c + E_a)/2$ (V) |
|---|---|---|---|
| **1** | −0.397 | −0.292 | −0.344 |
| **2** | −0.658 | −0.548 | −0.603 |
| **3a** | −1.150 | −0.689 | −0.919 |
| **3b** | −0.865 | −0.549 | −0.707 |
| **3c** | −1.013 | −0.720 | −0.867 |
| **3d** | −0.922 | −0.613 | −0.768 |
| **3e** | −1.024 | −0.549 | −0.787 |
| **4** | −1.059 | −0.497 | −0.769 |
| **5** | −1.077 | −0.469 | −0.773 |
| Menadione[d] | −0.755 | −0.601 | −0.678 |

[a] Measured in DMF–0.1 M TBAP on Pt as working and Ag–AgCl as reference electrodes, under $N_2$ atmosphere, at a scan rate of 100 mV/s.
[b] Cathodic peak potential.
[c] Anodic peak potential.
[d] Clinically used anticancer drug with a quinonoid structure.

## 2.2. Biological studies

### 2.2.1. Antitumor assessment in vivo

The compounds were assessed in vivo on the 16th day after transplantation with EAC tumor (Table 2). The increase in body weight for the 'untreated control' mice was nearly 6–8 g on an average, which was much less (∼2–4 g) for the groups treated with **3a**, **3b**, **3d**, **3e** and **5**. This would signify considerable enhancement in the inhibitory activity of most of the novel amino derivatives in comparison to their precursors (**1** and **2**), which was further corroborated by the significant increase in mean survival periods of all the 'treated groups' (ILS > 25%) [29]. In fact, the aminoacetate (**5**) exhibited the highest potency, the growth of tumor in this group being ∼60% less than that of the 'untreated control', and also in terms of maximum longevity (ILS = 93%). In fact, the mice treated with **5** displayed no toxic symptoms, either in external appearance, or in terms of general activity, such as intake of food and water. Also, the liver tissues collected from this group showed substantial restoration to normal pathological status, as compared to the markedly damaged hepatic cells seen in the liver sections of 'untreated controls' (Fig. 1). Thus, the dose regimen (1 mg $kg^{-1}$ $day^{-1}$; ip; five doses; alternate days) was fairly tolerable to the animals. This was also established from a separate study on single-dose acute toxicity ($LD_{50}$), which was determined for the compounds **1**, **2** and **5** to be approximately 25, 45 and >60 mg $kg^{-1}$ (ip), respectively, in 'normal' Swiss A mice [30]. Further corroboration was obtained through estimation of the liver function enzymes, LDH and AP, presented in Table 2, which showed that the treatment with diospyrin derivatives could effectively restore the enzyme activities to fairly 'normal' levels [14,31].

### 2.2.2. Antitumor assessment in vitro

The cytotoxicity of diospyrin (**1**) and its derivatives has been evaluated by MTT assay in EAC, A375 and Hep2 tumor cells, as well as in peripheral blood mononuclear cells (PBMC) [32]. Doxorubicin, a clinically used quinonoid anticancer agent, was taken as a positive control. The cytotoxicity induced by each of the compounds, cultured for 24 h with the respective cells, was expressed in terms of $IC_{50}$ and summarized in Table 3.

*2.2.2.1. EAC.* In EAC cells, the cytotoxicity of most of the amino analogues (except **3c**; Table 3) was greater than the

Table 2
Tumor-inhibitory effect of diospyrin (**1**) and its amino derivatives on Ehrlich ascites carcinoma (EAC) in Swiss A mice

| Drug[a] | Mean increase in tumor growth[b] ± S.E. on day '16' | | Increase in survival time[c] | | | Enzyme activity in blood serum[f] | |
|---|---|---|---|---|---|---|---|
| | | | MST[d] ± S.E. | | ILS[e] (%) | | |
| | Treated (T) | Control (C) | Treated (T) | Control (C) | | LDH[g,h] | AP[i,j] |
| **1** | 4.3 ± 1.2 | 6.5 ± 0.8 | 23.2 ± 3.0 | 17.0 ± 0.8 | 36 | 16.3 ± 0.7** | 3.7 ± 0.2** |
| **2** | 2.9 ± 0.7* | 6.5 ± 0.8 | 29.8 ± 7.5 | 19.0 ± 0.6 | 57 | 25.5 ± 1.6** | 4.9 ± 0.2** |
| **3a** | 2.7 ± 0.3** | 6.8 ± 0.2 | 34.3 ± 2.9 | 20.3 ± 1.8 | 69 | 25.5 ± 0.5** | 6.4 ± 0.1** |
| **3b** | 2.9 ± 0.4** | 6.8 ± 0.2 | 34.0 ± 3.1 | 20.3 ± 1.8 | 67 | 23.5 ± 1.4** | 5.4 ± 0.3** |
| **3c** | 3.0 ± 0.2** | 6.2 ± 0.3 | 28.4 ± 3.4 | 19.3 ± 2.3 | 47 | 31.8 ± 0.9** | 3.1 ± 0.1** |
| **3d** | 2.3 ± 0.2** | 6.2 ± 0.3 | 34.9 ± 2.8 | 19.3 ± 2.3 | 81 | 24.6 ± 1.7** | 5.9 ± 0.3** |
| **3e** | 3.4 ± 0.2** | 8.3 ± 0.6 | 31.5 ± 4.8 | 18.5 ± 1.7 | 72 | 34.7 ± 3.4** | 4.4 ± 0.2** |
| **4** | 3.9 ± 0.5** | 8.3 ± 0.6 | 30.0 ± 2.7 | 18.5 ± 1.7 | 62 | 37.5 ± 1.8** | 4.9 ± 0.2** |
| **5** | 3.3 ± 0.2** | 8.3 ± 0.6 | 35.7 ± 2.6 | 18.5 ± 1.7 | 93 | 26.3 ± 0.7** | 5.5 ± 0.1** |

*$p < 0.01$, and **$p < 0.001$ vs. respective C group, as determined by Student's *t*-test.
[a] Treated (T) group mice inoculated ip with quinonoids (1 mg $kg^{-1}$ $day^{-1}$) dissolved in 0.1 mL of DMSO on days 1, 3, 5, 7 and 9; control (C) group similarly inoculated with DMSO only.
[b] Number of mice in each group = 10.
[c] Number of mice in each group = 6.
[d] Median survival time (in days); mice surviving more than 60 days excluded from calculation of MST [29].
[e] Increase in life span (ILS) = [T/C − 1] × 100%; ILS > 25% regarded as significant inhibition of tumor growth in vivo [29].
[f] On day '16', blood serum collected from four mice to assess activity of LDH and AP enzymes [14].
[g] LDH unit = (ΔO.D. at 340 nm $min^{-1}$ $mg^{-1}$ protein) × $10^{-3}$.
[h] LDH of 'normal' group = 8.8 ± 0.8 and 'control' group = 173.4 ± 4.2.
[i] AP unit = micromoles of PNPP hydrolysed per minute per decilitre of serum.
[j] AP of 'normal' group = 8.4 ± 0.3 and 'control' group = 1.2 ± 0.2.

precursors **1** and **2**, and also found to be more or less concordant with the in vivo trend (vide Table 2). For example, the $IC_{50}$ values of **3d** and **5** (0.09 and 0.06 μM, respectively) indicated a dramatic enhancement in cytotoxicity, while the same compounds also showed the maximum tumor inhibition in vivo (ILS = 81 and 93%, respectively).

*2.2.2.2. A375 cells.* In A375 cells, the amino derivatives, except **3e** and **4**, showed enhanced cytotoxicity than **1** (the natural product). However, only two of them, viz. **3b** and **5** with $IC_{50}$ values of 0.08 and 0.06 μM, respectively, exhibited nearly 3-fold improvement when compared to their synthon, **2** ($IC_{50}$ = 0.20 μM). Incidentally, in another study [33], synthesis of several amine-anchored glycoconjugates of **2** had been undertaken, resulting in effective modification of the antiproliferative activity in this cell line when the aminophenyl mannosyl adduct showed a 10-fold reduction in $IC_{50}$ value (0.02 μM).

*2.2.2.3. Hep2 cells.* As given in Table 3, the compounds **3a**, **3d** and **5** were observed to be significantly more cytotoxic than **1** in Hep2 cells, while none of the amino-diospyrins proved to be more effective than **2** ($IC_{50}$ = 0.39 μM). However, the aminophenyl mannosyl adduct as mentioned above was reported to show a marginal improvement in this cell line, with $IC_{50}$ value of 0.26 μM [33].

*2.2.2.4. PBMC.* From a comparative assessment in PBMC (Table 3), the therapeutic prospect of diospyrin series was quite obvious, as at least six of the quinonoids showed $IC_{50} > 70$ μM. Thus, barring two of them (**3a** and **4**), the amination of **2** proved to be significantly beneficial in this regard. The most notable derivative was the aminoacetate **5**, which exhibited ~100- to 1500-fold cytotoxicity against the tumor cells as compared to PBMC. Further, the $LD_{50}$ data, as mentioned in Section 2.2.1, could also corroborate the markedly low toxicity of **5** observed in vivo when inoculated in normal mouse. In fact, the toxicity of all diospyrin derivatives in normal human lymphocytes was much less in comparison to the standard drug, viz. doxorubicin ($IC_{50}$ = 15.5 μM; Table 3).

### 2.2.3. ROS generation in EAC cells

While attempting to elucidate the mechanism of the antitumor action of **1** and its derivatives, it has already been shown that alkyl derivatives of **1** could induce apoptosis in human cancer cell lines through involvement of ROS-mediated pathway, revealing dramatic changes in mitochondrial transmembrane potential and other associated events signaling cell death [16–18,34]. Presumably, the cytotoxicity of quinonoid compounds would be proportionate to the oxidative stress through generation of ROS via one-electron reduction [22]. Therefore, it is to be expected that introducing vicinal oxidizable groups in the quinonoid skeleton would create additional redox-active centres leading to enhanced bioactivation through facile formation of the semiquinone radicals [35]. This prompted us to evaluate each of the aforesaid quinonoids for its ability to generate ROS in tumor cells using DCFH-DA, a fluorescent probe [17]. For this study, EAC cells were selected since most of the amino analogues were found to show enhanced cytotoxicity in EAC in comparison to the synthon, **2**. It was found that ROS was generated in a dose-dependent manner (data not shown). The R.F.I. values of **1** and its derivatives, presented in Fig. 2, were determined at a given concentration of 0.4 μM in order to make a comparative assessment. Thus, **3d** and **5** were most effective, producing

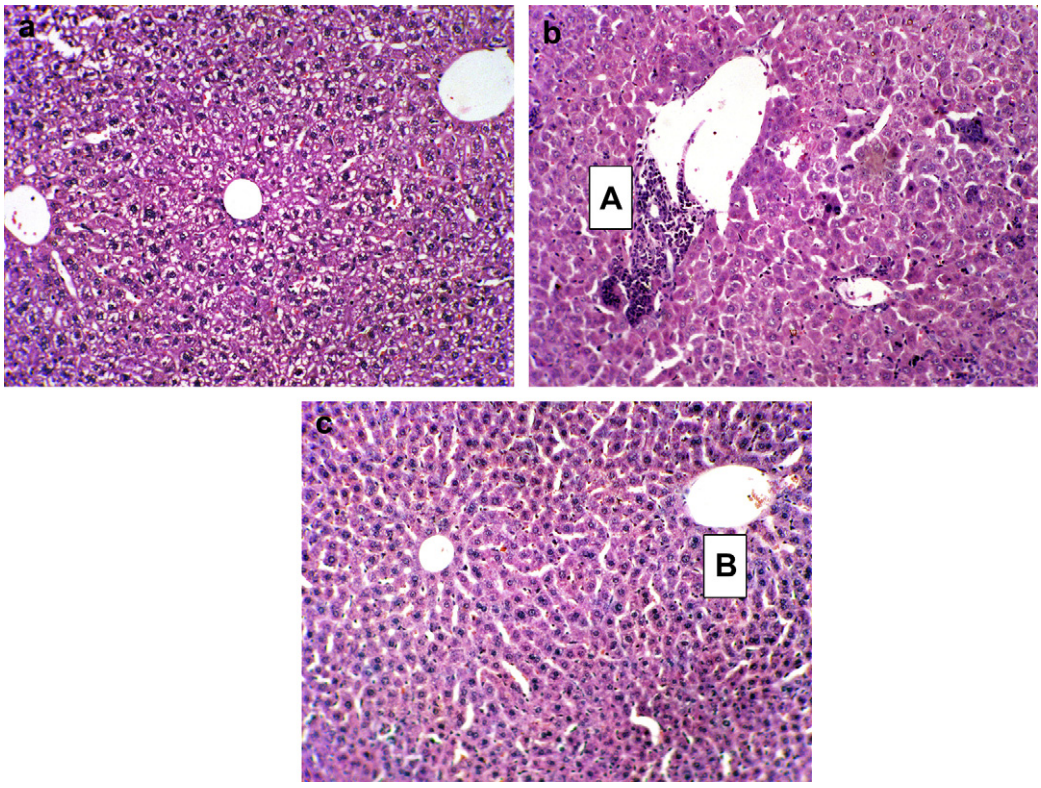



Fig. 1. (a) Photomicrograph [250×] of histopathological study of liver from 'normal' Swiss A mouse. (b) Photomicrograph [250×] of histopathological study of liver from Swiss A mouse bearing EAC tumor (16 days) showing damaged cellular architecture, infiltration of lymphocytes and marked dilatation of central veins. Dilated sinusoids lined by hyperactive Kupffer cells are indicated by (A). (c) Photomicrograph [250×] of histopathological study of liver from Swiss A mouse bearing EAC tumor (16 days) and treated with compound **5**. Dilated sinusoid with minimum infiltration of lymphocytes and nearly normal arrangement of hepatic cell chords are indicated by (B).

9- to 10-fold increase in the fluorescence intensity in comparison to the 'untreated control' cells. Again, a parallel experiment with addition of NAC, a specific scavenger for $H_2O_2$, showed dramatic reduction in fluorescence intensity, almost to the level of the 'untreated control' [36]. This could confirm the formation of $H_2O_2$ as the predominant ROS generated by **1** and its derivatives, as well as by menadione, a clinical agent with quinonoid structure used in cancer chemotherapy [37].

Table 3
Evaluation of cytotoxicity towards tumor cells and PBMC by diospyrin and its derivatives

| Compound | $IC_{50}$ (μM) ± S.E.[a,b] | | | |
|---|---|---|---|---|
| | EAC | A375 | Hep2 | PBMC |
| **1** | 0.84 ± 0.01 | 0.82 ± 0.03 | 3.58 ± 0.56 | 78.32 ± 3.41 |
| **2** | 0.65 ± 0.02** | 0.20 ± 0.02*** | 0.39 ± 0.03** | 53.26 ± 3.80 |
| **3a** | 0.25 ± 0.01*** | 0.63 ± 0.12 | 0.65 ± 0.28** | 44.92 ± 2.08 |
| **3b** | 0.24 ± 0.04*** | 0.08 ± 0.02*** | 2.04 ± 0.08 | 73.76 ± 4.15 |
| **3c** | 1.41 ± 0.07 | 0.55 ± 0.04** | 2.61 ± 0.20 | >100.0 |
| **3d** | 0.09 ± 0.01*** | 0.57 ± 0.01** | 0.86 ± 0.18** | 96.17 ± 4.24 |
| **3e** | 0.35 ± 0.02 | 3.24 ± 0.07 | 3.51 ± 0.62 | >100.0 |
| **4** | 0.37 ± 0.03*** | 1.93 ± 0.05 | 2.34 ± 0.47 | 40.63 ± 3.23 |
| **5** | 0.06 ± 0.02*** | 0.06 ± 0.01*** | 0.92 ± 0.13** | 86.46 ± 4.91 |
| **6**[c] | 0.58 ± 0.01** | 1.07 ± 0.04 | 2.39 ± 0.84 | 55.49 ± 0.32 |
| Doxorubicin[d] | >10.0 | 0.01 ± 0.01 | 0.42 ± 0.04 | 15.51 ± 1.72 |

*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, determined by Student's *t*-test, indicate significant enhancement of tumor-inhibitory activity with respect to compound **1**.

[a] Inhibitory concentration to reduce 50% cell growth evaluated by MTT assay.

[b] Data represent mean values (±S.E.) for three independent determinations.

[c] $IC_{50}$ data reproduced from Das Sarma et al. [19].

[d] Anticancer drug as 'standard clinical agent'.

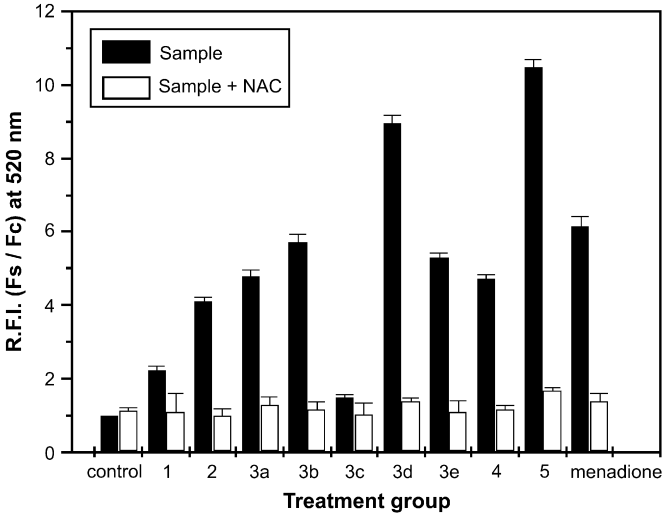



Fig. 2. ROS generation, in vitro, in EAC cells ($2 \times 10^5$/mL) treated with quinonoids (0.4 μM) in the presence or absence of pre-incubation with NAC (100 μM), followed by incubation with DCFH-DA (10 μM) for 20 min at 37 °C. R.F.I. = relative fluorescence intensity of sample ($F_s$) with respect to control ($F_c$). Error bars represent the standard error in each group ($n = 3$).

On critical examination of the data presented in Fig. 2, it appears that the order of ROS generation (R.F.I.) by the quinonoid derivatives could be correlated, more or less, with their in vitro cytotoxicity ($IC_{50}$) in EAC cells. For example, a decrease in the $IC_{50}$ value, for **3e** and **4**, was observed after introducing the free $-NH_2$ group(s) in **2**, with a concomitant increase in R.F.I. A similar trend followed in **3a** and **3b** by substituting one of the amino protons with an aromatic group, while substitution with $-CH_2CO_2Et$/$-COCH_3$ caused dramatic changes in **3d** and **5**, respectively, with concomitant enhancement in their biological efficacy against the tumor model in vitro as well as in vivo. Again, it is interesting to note that by merely preceding these type of substituents by a $-CH_2$-spacer resulted in drastic reduction in the activity, for example in **3c**, both in terms of cytotoxicity and ROS generation, bringing down their potency even lower than that of **1** and **2**. Evidently, the delocalisation effect of the amino groups could partially account for the biological phenomenon.

Again, it is to be expected that the redox status of the cellular system would be modulated by ROS. Since the ease of ROS generation through reduction of a quinonoid would depend on its electrochemical parameters, the redox potential of a quinone would influence its overall biological profile, which encompasses the functional, toxicological, mutagenic and antitumor activities. With this view, the redox potential of these naphthoquinonoids was determined by cyclic voltammetry using DMF, an aprotic solvent, which mimics the environment of cell membrane [22]. A quinone with higher reduction potential would favour the formation of its semiquinone radical more readily leading to the generation of ROS. However, the observations from the biological experiments do not show absolute corroboration with this hypothesis. From Table 1, it was found that diospyrin (**1**) has a much less negative $E_{1/2}$ value than that of its dimethyl ether derivative (**2**) as well as its amino analogues, hence, **1** would be expected to generate more ROS than its congeners. On the contrary, most of the derivatives were more cytotoxic than **1** and exhibited greater antitumor activity, both in vivo and in vitro (Tables 2 and 3). Evidently electrochemical parameters of quinonoids, although essential for analysis of cellular events, do not always show quantitative correlation with their cytotoxic properties. In fact, many other factors like lipophilicity, membrane permeability, site-specific binding, etc., need critical consideration in order to rationalize the relationship of structural modulation and cytotoxicity [38].

## 3. Conclusion

Starting with diospyrin (**1**), a plant-derived quinonoid, a series of novel amino derivatives were synthesized in fairly good yield, using appropriate aminating agents. In comparison to **1**, the inhibitory activity of most of these analogues was found to be significantly improved both in vivo and in vitro against a murine tumor model (EAC), while a variable enhancement in activity could be observed in two human cancer cell lines, viz. A375 and Hep2. Further, it was evident from the ratio of respective $IC_{50}$ values that the cytotoxicity of diospyrin and its derivatives towards normal human lymphocytes was markedly lower (between 17- and 1441-fold) as compared to the tumor cells. Thus, from the present study, an aminoacetate derivative (**5**) was found to show the maximum therapeutic prospect, and would act as a novel 'lead' for the development of effective antiproliferative agents, most probably through appropriate substitution in the *N*-acetyl moiety.

Table 4
Evaluation of cytotoxicity against human cancer cell lines by diospyrin and its alkyl ether derivatives

| Compound | $IC_{50}$ (μM) ± S.E.[a,b] | | | |
|---|---|---|---|---|
| | HL-60[c] | K-562[c] | MCF-7[d] | A431[e] |
| **1** | >100 | >100 | 74.78 ± 3.12 | 65.32 ± 5.76 |
| **2** | 64.14 ± 3.86 | >100 | 46.42 ± 4.19 | 28.17 ± 4.54 |
| **6** | 30.07 ± 1.94 | 40.16 ± 3.58 | 18.95 ± 1.05 | 15.90 ± 4.54 |

[a] Inhibitory concentration to reduce 50% cell growth evaluated by MTT assay.
[b] Data represent mean values (±S.E.) for three independent determinations.
[c] Data reproduced from Chakraborty et al. [16].
[d] Data reproduced from Hazra et al. [17].
[e] Data reproduced from Ph.D. dissertation of Binod Kumar [34].

In order to carry out further modification of the diospyrin template, some of the recent observations also need to be taken into cognizance [16–19,34]. It was already noted that alkylation of the two phenolic –OH groups could produce a dramatic increase in cytotoxicity, the ethyl substitution (**6**; Scheme 1) being more effective than the methyl analogue (**2**), presumably due to the relative difference in polarity and membrane permeability. The $IC_{50}$ data for **2** and **6** (Table 3), taken together with Table 4, which give a consolidated outcome from earlier studies in seven types of tumor cells [16–19,34] would support this view, although the $IC_{50}$ of **6** in A375 cells did not exactly conform to this speculation. However, the introduction of an ethanolamine moiety in **6** generated a potent inhibitor of the same cell line ($IC_{50} = 0.07$ μM), which was nearly 50-fold more cytotoxic in comparison to its dimethyl analogue ($IC_{50} = 3.18$ μM) obtained from **2** [19]. Thus, suitable amino substitution at 3′-position of either **2** or **6**, followed by screening against a variety of human cell lines, might lead to a good therapeutic candidate against a specific tumor strain. Again, a di-epoxide derivative of **2** also exhibited remarkable antiproliferative activity in several tumor cells with substantially lower toxicity in normal PBMC [19]. Thus, other nucleophilic additions in diospyrin template might also be considered in future. Obviously, this would require a large amount of the scarce natural product, pending suitable modification of the synthetic procedure to achieve the desired yield of diospyrin.

## 4. Experimental

### 4.1. Chemistry

Glycine ethyl ester hydrochloride and β-naphthylamine were purchased from Sigma Chemical Company, USA; ethanolamine was procured from BDH Chemicals Ltd, Poole, England. All other reagents and solvents used were obtained

from Sisco Research Laboratory, India. Column chromatography was performed on silica gel (60–120 mesh) and preparative TLC on 20 cm × 20 cm glass plates coated with a 2 mm layer of silica gel G from Merck, India. Petroleum ether was used in the boiling range of 60–80 °C. All organic solvents were distilled prior to use. Melting points were determined on Toshniwal melting point apparatus (Cat no: CL-0301) and are uncorrected. UV–vis absorption spectra were recorded with Shimadzu UV 1601 spectrophotometer. IR spectra were obtained on a Perkin Elmer Spectrum RX I FT-IR system in KBr pellets. The $^1$H and $^{13}$C NMR spectra were recorded on a Bruker AM 300L Supercon NMR spectrometer operating at 300.13 and 75.47 MHz, respectively. Chemical shifts were expressed in parts per million ($\delta$) downfield relative to internal reference $Me_4Si$ and $J$ values were reported in hertz (Hz). The splitting pattern abbreviations in $^1$H spectra are as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br s = broad singlet. EI-MS were run on an AEI MS902 spectrometer equipped with an MSS data acquisition system, version 10 (Mass Spectrometer Services, Manchester, UK) and ESI-MS was run on WATERS Micromass Q-Tof microinstrument. Elemental analyses were carried out on a Perkin Elmer instrument 2400 Series II CHN analyzer. Results obtained were within ±0.4% of the theoretical value. Analytical RPHPLC was performed on a μBondapak $C_{18}$ steel column (30 cm × 0.39 cm i.d.; particle size 10 μm), with an UV–vis variable wavelength detector set at 255 nm. Elution was carried out with isocratic mobile phase acetonitrile–water (75:25, v/v) at a flow rate of 1.0 mL/min at ambient temperature.

### 4.1.1. Synthesis of amino derivatives of diospyrin

*4.1.1.1. 3′-(4-Chloroanilinyl) diospyrin dimethyl ether (**3a**).* A mixture of diospyrin dimethyl ether (**2**, 100 mg, 0.25 mmol) in chloroform (3 mL) and *p*-chloroaniline (64 mg, 0.5 mmol) in ethanol (1 mL) was refluxed at 80 °C for 10 h. The reaction mixture was diluted with dichloromethane (10 mL) and washed with water (3 × 10 mL). The pooled organic layer was dried over anhydrous sodium sulphate and the solvent was removed to get a crude product, which was chromatographed over silica gel. Elution with petroleum ether–ethyl acetate = 7:3, v/v, gave the desired product **3a** (110 mg, 80%) as a reddish orange powder (dichloromethane–petroleum ether), mp 278 °C. TLC $R_f$ 0.53 (petroleum ether–ethyl acetate = 9:11, v/v). UV ($CHCl_3$): $\lambda_{max}$ (log $\varepsilon$) 271.5 nm (3.20). IR (KBr): $\nu_{max}$ ($cm^{-1}$) 3323, 2933, 1656. $^1$H NMR ($CDCl_3$): $\delta$ 2.30 (3H, s, 7′-$CH_3$), 2.50 (3H, s, 7-$CH_3$), 3.70 (3H, s, 5′-$OCH_3$), 4.03 (3H, s, 5-$OCH_3$), 6.30 (1H, s, H-2′), 6.80 (1H, s, H-3), 7.17 (1H, s, H-6), 7.23 (2H, d, $J$ = 9.0 Hz, H-2″ and H-6″), 7.39 (2H, d, $J$ = 9.0 Hz, H-3″ and H-5″), 7.61 (1H, s, H-8), 7.65 (1H, br s, NH), 7.86 (1H, s, H-8′). $^{13}$C NMR ($CDCl_3$): $\delta$ 20.9 (7′-$CH_3$), 22.3 (7-$CH_3$), 56.4 (5-$OCH_3$), 62.2 (5′-$OCH_3$), 102.5 (C-2′), 117.6 (C-4a), 118.5 (C-6), 120.0 (C-4′a), 120.6 (C-8), 123.9 (C-2″, C-6″), 124.2 (C-8′), 129.7 (C-3″, C-5″), 130.8 (C-4″), 133.9 (C-6′), 134.0 (C-8′a), 134.9 (C-3′), 136.0 (C-1″), 139.9 (C-3), 143.3 (C-8a), 145.3 (C-7′), 146.1 (C-7), 146.7 (C-2), 158.5 (C-5′), 159.9 (C-5), 179.4 (C-4′), 182.9 (C-1′), 183.2 (C-4), 184.0 (C-1). RPHPLC $R_t$ 4.97 min (acetonitrile–water = 75:25, v/v). MS (EI, *m/z*, relative intensity, %) 528 (10.2) [M + 1], 527 (12.9) [$M^+$], 497 (7.9), 402 (25.1), 374 (8.4), 372 (18.4), 357 (29.6), 331 (17.4), 315 (14.8), 246 (10.8), 228 (10.9), 113 (14.8), 91 (64.7), 89 (66.7), 43 (100). Anal. ($C_{30}H_{22}O_6NCl$) C, H, N.

*4.1.1.2. 3′-(2-Naphthylamino) diospyrin dimethyl ether (**3b**).* β-Naphthylamine (54 mg, 0.38 mmol) was added slowly to a stirred solution of **2** (80 mg, 0.2 mmol) in chloroform (2 mL) and ethanol (2 mL). The mixture was vigorously stirred at room temperature for 6 days and then concentrated in vacuo. The resulting solid residue was subjected to preparative TLC in the solvent mixture chloroform–ethyl acetate–petroleum ether = 8:1:0.5 (v/v/v), followed by crystallization from dichloromethane–diethyl ether, to obtain **3b** as a dark orange red solid (94 mg, 88%), mp 240 °C. TLC $R_f$ 0.51 (petroleum ether–ethyl acetate = 1:1, v/v). UV ($CHCl_3$): $\lambda_{max}$ (log $\varepsilon$) 260 nm (4.69), 285 nm (4.58), 394 nm (3.93). IR (KBr): $\nu_{max}$ ($cm^{-1}$) 3270, 2925, 1656. $^1$H NMR ($CDCl_3$): $\delta$ 2.31 (3H, s, 7′-$CH_3$), 2.51 (3H, s, 7-$CH_3$), 3.74 (3H, s, 5′-$OCH_3$), 4.05 (3H, s, 5-$OCH_3$), 6.57 (1H, s, H-2′), 6.82 (1H, s, H-3), 7.16 (1H, s, H-6), 7.37 (1H, d, $J$ = 8.6 Hz, H-3″), 7.48 (1H, t, $J$ = 7.1 Hz, H-6″), 7.53 (1H, t, $J$ = 7.3 Hz, H-7″), 7.63 (1H, s, H-8), 7.74 (1H, br s, H-1″), 7.82 (1H, d, $J$ = 6.8 Hz, H-8″), 7.84 (1H, d, $J$ = 6.9 Hz, H-5″), 7.89 (1H, d, $J$ = 9.7 Hz, H-4″), 7.91 (1H, s, NH), 7.91 (1H, s, H-8′). $^{13}$C NMR ($CDCl_3$): $\delta$ 20.9 (7′-$CH_3$), 22.3 (7-$CH_3$), 56.4 (5-$OCH_3$), 62.4 (5′-$OCH_3$), 102.4 (C-2′), 117.6 (C-4a), 118.4 (C-6), 119.3 (C-1″), 120.1 (C-4′a), 120.6 (C-8), 121.6 (C-3″), 124.3 (C-8′), 125.8 (C-6″), 127.0 (C-7″), 127.4 (C-5″), 127.7 (C-8″), 129.6 (C-4″), 131.0 (C-4″a), 133.8 (C-8′a), 133.9 (C-2″), 133.9 (C-1″a), 134.9 (C-6′), 135.0 (C-3′), 139.9 (C-3), 143.4 (C-8a), 145.2 (C-2), 146.0 (C-7), 146.6 (C-7′), 158.5 (C-5′), 159.8 (C-5), 179.6 (C-4′), 183.1 (C-1′), 183.3 (C-4), 184.0 (C-1). RPHPLC $R_t$ 4.12 min (acetonitrile–water = 75:25, v/v). ESI-MS: 544 (M + H), 566 (M + Na). Anal. ($C_{34}H_{25}O_6N$) C, H, N.

*4.1.1.3. 3′-Benzylamino diospyrin dimethyl ether (**3c**).* To an ice-cold solution of diospyrin dimethyl ether (**2**, 80 mg, 0.2 mmol) in chloroform (2 mL), freshly distilled benzyl amine (50 μL, 0.46 mmol) in ethanol (1 mL) and chloroform (1 mL) were added slowly. The reaction mixture was stirred at 0–5 °C for 90 min. The solution was diluted with chloroform (5 mL) and treated with cold dilute HCl (0.1 N, 10 mL). The organic layer was washed with water (3 × 10 mL), dried over anhydrous sodium sulphate and evaporated to dryness under vacuum to get the crude product. It was purified by column chromatography over silica gel using petroleum ether–ethyl acetate (3:1, v/v) as the eluent and the product, **3c**, was crystallized from a mixture of acetone and diethyl ether as an orange-yellow powder (84 mg, 83%), mp 272 °C. TLC $R_f$ 0.48 (petroleum ether–ethyl acetate = 1:1, v/v). UV–vis ($CHCl_3$): $\lambda_{max}$ (log $\varepsilon$) 216 nm (4.04), 233 nm (3.92), 269 nm (3.89), 408 nm (3.29). IR (KBr):

$\nu_{max}$ ($cm^{-1}$) 3335, 2930, 1658. $^1H$ NMR ($CDCl_3$): $\delta$ 2.30 (3H, s, 7′-$CH_3$), 2.50 (3H, s, 7-$CH_3$), 3.69 (3H, s, 5′-$OCH_3$), 4.03 (3H, s, 5-$OCH_3$), 4.37 (2H, d, $J = 5.7$ Hz, NHC$H_2$Ph), 5.76 (1H, s, H-2′), 6.34 (1H, t, $J = 5.6$ Hz, N$H$C$H_2$Ph), 6.78 (1H, s, H-3), 7.15 (1H, s, H-6), 7.30–7.42 (5H, m, H-2″, H-3″, H-4″, H-5″, H-6″), 7.60 (1H, s, H-8), 7.87 (1H, s, H-8′). $^{13}C$ NMR ($CDCl_3$): $\delta$ 21.3 (7′-$CH_3$), 22.8 (7-$CH_3$), 47.3 (NH$CH_2$), 56.9 (5-$OCH_3$), 62.7 (5′-$OCH_3$), 101.0 (C-2′), 118.1 (C-4a), 118.9 (C-6), 120.7 (C-4′a), 121.1 (C-8), 124.7 (C-8′), 128.0 (C-2″ and C-6″), 128.5 (C-4″), 129.4 (C-3″ and C-5″), 134.0 (C-8a), 134.4 (C-6′), 135.4 (C-8′a), 136.3 (C-3′), 140.4 (C-3), 143.9 (C-2), 146.3 (C-7′), 147.1 (C-7), 148.9 (C-1″a), 158.8 (C-5′), 160.3 (C-5), 180.0 (C-4′), 182.6 (C-1′), 183.8 (C-4), 184.5 (C-1). RPHPLC $R_t$ 4.53 min (acetonitrile–water = 75:25, v/v). MS (EI, *m/z*, relative intensity, %) 504 (100) [M − 3], 489 (34), 475 (11), 417 (4), 306 (1), 230 (0.5), 177 (1.5), 91 (40). Anal. ($C_{31}H_{25}O_6N$) C, H, N.

*4.1.1.4. 3′-(Ethylglycinato) diospyrin dimethyl ether (**3d**).* Diospyrin dimethyl ether (**2**, 100 mg, 0.25 mmol) in chloroform (2 mL) was added slowly to an ice-cold solution of glycine ethyl ester hydrochloride (70 mg, 0.5 mmol) in a mixture of chloroform (2 mL), ethanol (1 mL) and sodium acetate (41 mg, 0.5 mmol). The solution was stirred at 0–5 °C for 3 h. It was diluted with dichloromethane (5 mL), washed with brine solution (1 × 15 mL), and the aqueous layer was extracted with dichloromethane (3 × 10 mL). The combined organic layer was washed with water (1 × 15 mL), dried over anhydrous sodium sulphate and finally concentrated to dryness. The crude residue was chromatographed on silica gel column. The desired compound (**3d**) was eluted with chloroform–ethyl acetate = 4:1, v/v, and crystallized from a mixture of acetone and diethyl ether as a brownish yellow powder (80 mg, 80%), mp 238 °C. TLC $R_f$ 0.45 (petroleum ether–ethyl acetate = 1:1). UV–vis ($CHCl_3$): $\lambda_{max}$ (log $\varepsilon$) 261 nm (4.42), 404 nm (3.78). IR (KBr): $\nu_{max}$ ($cm^{-1}$) 3308, 2931, 1662. $^1H$ NMR ($CDCl_3$): $\delta$ 1.31 (3H, t, $J = 7.1$ Hz, −NHC$H_2$CO$_2$CH$_2$C$H_3$), 2.27 (3H, s, 7′-$CH_3$), 2.48 (3H, s, 7-$CH_3$), 3.67 (3H, s, 5′-$OCH_3$), 3.91 (2H, d, $J = 5.23$ Hz, −NHC$H_2$CO$_2$CH$_2$CH$_3$), 4.02 (3H, s, 5-$OCH_3$), 4.28 (2H, q, $J = 7.13$ Hz, NHCH$_2$CO$_2$C$H_2$CH$_3$), 5.61 (1H, s, H-2′), 6.52 (1H, t, $J = 5.12$ Hz, −NH), 6.77 (1H, s, H-3), 7.14 (1H, s, H-6), 7.58 (1H, s, H-8), 7.84 (1H, s, H-8′). $^{13}C$ NMR ($CDCl_3$): $\delta$ 14.1 (CO$_2$CH$_2$C$H_3$), 20.8 (7′-$CH_3$), 22.2 (7-$CH_3$), 44.1 (NH$CH_2$), 56.5 (5-$OCH_3$), 62.1 (CO$_2$C$H_2$CH$_3$), 62.3 (5′-$OCH_3$), 100.9 (C-2′), 118.0 (C-4a), 118.7 (C-6), 120.4 (C-4′a), 120.7 (C-8), 124.2 (C-8′), 133.9 (C-6′), 134.2 (C-8′a), 135.4 (C-3′), 140.1 (C-3), 143.6 (C-8a), 145.8 (C-7′), 146.6 (C-7), 148.2 (C-2), 158.6 (C-5′), 160.0 (C-5), 168.2 (C$O_2$CH$_2$CH$_3$), 178.9 (C-4′), 182.2 (C-1′), 183.2 (C-4), 184.1 (C-1). RPHPLC $R_t$ 4.17 min (acetonitrile–water = 75:25, v/v). ESI-MS: 504 (M + H), 526 (M + Na). Anal. ($C_{28}H_{25}O_8N$) C, H, N.

*4.1.1.5. 3′-Amino diospyrin dimethyl ether (**3e**) and 3,3′-diamino diospyrin dimethyl ether (**4**).* Diospyrin dimethyl ether (**2**, 80 mg, 0.2 mmol) in chloroform (2 mL) was added slowly to the stirred ice-cold solution of hydroxylamine hydrochloride (35 mg, 0.5 mmol) in a mixture of chloroform (2 mL), ethanol (1 mL) and triethylamine (70 μL, 0.5 mmol). The solution was stirred at 0–5 °C for 1 h. The reaction mixture was diluted with dichloromethane (5 mL), washed with brine solution (1 × 15 mL), and the aqueous layer was extracted with dichloromethane (3 × 10 mL). The combined organic phase was washed with water (1 × 15 mL), dried over anhydrous sodium sulphate and finally the solvent was evaporated to get a crude product. It was chromatographed on silica gel column using a mixture of chloroform and ethyl acetate as eluent. Two products were isolated, viz., a monoamine (**3e**) fraction, from chloroform–ethyl acetate = 5:1 (v/v), which was crystallized from dichloromethane–petroleum ether as a golden yellow powder (55 mg, 66%), mp 294 °C, and a diamine (**4**), from the chloroform–ethyl acetate = 1:4 (v/v), fraction followed by crystallization from acetone–diethyl ether as an orange powder (11 mg, 12.5%), mp 302 °C. Compound **3e**: TLC $R_f$ 0.53 (petroleum ether–ethyl acetate = 2:3, v/v). UV–vis ($CHCl_3$): $\lambda_{max}$ (log $\varepsilon$) 218 nm (4.49), 264 nm (4.42), 301 nm (4.03), 407 nm (3.81). IR (KBr): $\nu_{max}$ ($cm^{-1}$) 3436, 3315, 2932, 1631. $^1H$ NMR ($CDCl_3$): $\delta$ 2.28 (3H, s, 7′-$CH_3$), 2.51 (3H, s, 7-$CH_3$), 3.69 (3H, s, 5′-$OCH_3$), 4.04 (3H, s, 5-$OCH_3$), 5.25 (2H, br s, exchangeable with $D_2O$, 3′-$NH_2$), 5.97 (1H, s, H-2′), 6.78 (1H, s, H-3), 7.16 (1H, s, H-6), 7.61 (1H, s, H-8), 7.86 (1H, s, H-8′). $^{13}C$ NMR (DMSO-$d_6$): $\delta$ 20.8 (7′-$CH_3$), 22.1 (7-$CH_3$), 56.8 (5-$OCH_3$), 62.2 (5′-$OCH_3$), 101.4 (C-2′), 117.3 (C-4a), 119.9 (C-6), 120.1 (C-8), 120.5 (C-4′a), 123.4 (C-8′), 133.6 (C-6′), 133.7 (C-8′a), 135.6 (C-3′), 140.2 (C-3), 143.3 (C-8a), 145.8 (C-7′), 147.8 (C-7), 151.9 (C-2), 157.9 (C-5′), 159.9 (C-5), 180.2 (C-4′), 181.5 (C-1′), 183.1 (C-4), 184.3 (C-1). RPHPLC $R_t$ 4.34 min (acetonitrile–water = 75:25, v/v). MS (EI, *m/z*, relative intensity, %) 417 (100) [$M^+$], 402 (52), 387 (32), 374 (26), 358 (14), 289 (6), 228 (10), 177 (18), 149 (15), 90 (29). Anal. ($C_{24}H_{19}O_6N$) C, H, N.

Compound **4**: TLC $R_f$ 0.43 (chloroform–ethyl acetate = 1:4, v/v). UV ($CHCl_3$): $\lambda_{max}$ (log $\varepsilon$) 214 nm (4.05), 228 nm (4.09), 272 nm (4.08), 297 nm (3.86), 384 nm (3.84). IR (KBr): $\nu_{max}$ ($cm^{-1}$) 3382, 2925, 1622. $^1H$ NMR ($CDCl_3$): $\delta$ 2.25 (3H, s, 7′-$CH_3$), 2.48 (3H, s, 7-$CH_3$), 3.70 (3H, s, 5′-$OCH_3$), 4.04 (3H, s, 5-$OCH_3$), 5.25 (4H, br s, exchangeable with $D_2O$, 3-$NH_2$ and 3′-$NH_2$), 5.91 (1H, s, H-2′), 7.04 (1H, s, H-6), 7.65 (1H, s, H-8), 7.84 (1H, s, H-8′). $^{13}C$ NMR (DMSO-$d_6$): $\delta$ 20.1 (7′-$CH_3$), 22.1 (7-$CH_3$), 56.5 (5-$OCH_3$), 61.3 (5′-$OCH_3$), 100.9 (C-2′), 107.9 (C-2), 115.7 (C-4a), 117.2 (C-6), 119.3 (C-8), 120.9 (C-4′a), 123.2 (C-8′), 133.4 (C-8′a), 134.8 (C-6′), 135.2 (C-3′), 143.3 (C-8a), 147.6 (C-7′), 148.2 (C-7), 151.6 (C-3), 159.2 (C-5′), 160.0 (C-5), 178.8 (C-4′), 179.7 (C-1′), 180.3 (C-4), 181.7 (C-1). RPHPLC $R_t$ 3.96 min (acetonitrile–water = 75:25, v/v). MS (EI, *m/z*, relative intensity, %) 432 (100) [$M^+$], 417 (51), 402 (94), 387 (13), 356 (8), 328 (6), 230 (4), 208 (5), 149 (6), 90 (11), 51 (4). Anal. ($C_{24}H_{20}O_6N_2$) C, H, N.

*4.1.1.6. 3′-(N-Acetylamino) diospyrin dimethyl ether (**5**).* Acetyl chloride (0.4 mL) was slowly added to an ice-cold solution of **3e** (50 mg, 0.12 mmol) in dry pyridine (1 mL). The mixture was vigorously stirred at room temperature for 30 min. It

was then treated with ice-cold dilute HCl (0.5%, 10 mL) and extracted with dichloromethane (10 mL). The organic layer was washed with water (2 × 15 mL) and dried over anhydrous sodium sulphate. After removing the solvent, the residue was chromatographed using petroleum ether–chloroform = 1:1, v/v, to furnish the acetate derivative (**5**), which was crystallized from dichloromethane–petroleum ether as a golden yellow powder (43 mg, 78%), mp 308 °C. TLC $R_f$ 0.59 (petroleum ether–chloroform = 1:4, v/v). UV–vis ($CHCl_3$): $\lambda_{max}$ (log $\varepsilon$) 221 nm (4.32), 260 nm (4.30), 301 nm (3.94), 407 nm (3.69). IR (KBr): $\nu_{max}$ ($cm^{-1}$) 3286, 2923, 1657, 1590, 1496, 1347, 1259, 1036. $^1$H NMR ($CDCl_3$): $\delta$ 2.28 (3H, s, 7′-$CH_3$), 2.29 (3H, s, NHCOC$H_3$), 2.51 (3H, s, 7-$CH_3$), 3.69 (3H, s, 5′-$OCH_3$), 4.04 (3H, s, 5-$OCH_3$), 6.78 (1H, s, H-3), 7.17 (1H, s, H-6), 7.61 (1H, s, H-8), 7.82 (1H, s, H-2′), 7.86 (1H, s, H-8′), 8.47 (1H, s, N$H$COCH$_3$). $^{13}$C NMR ($CDCl_3$): $\delta$ 20.9 (7′-$CH_3$), 22.4 (7-$CH_3$), 25.1 (NHCO$CH_3$), 56.5 (5-$OCH_3$), 62.4 (5′-$OCH_3$), 115.8 (C-2′), 117.7 (C-4a), 118.6 (C-6), 119.8 (C-4′a), 120.7 (C-8), 124.5 (C-8′), 133.8 (C-6′), 133.9 (C-8′a), 135.1 (C-3′), 140.1 (C-3), 140.6 (C-8a), 143.2 (C-7), 146.3 (C-7′), 146.8 (C-2), 158.6 (C-5′), 159.9 (C-5), 169.4 (CONH), 178.8 (C-4′), 183.2 (C-1′), 184.0 (C-4), 184.8 (C-1). HPLC $R_t$ 4.25 min (acetonitrile–water = 75:25, v/v). MS (EI, *m/z*, relative intensity, %) 457 (100) [M − 2], 443 (18), 428 (23), 416.5 (55), 402 (42), 387 (12), 372 (13), 358 (11), 289 (1), 228 (7), 177 (10), 149 (9), 90 (16), 43 (47). Anal. ($C_{26}H_{21}O_7N$) C, H, N.

#### 4.1.2. Cyclic voltammetric studies

Cyclic voltammetry was performed with the electrochemical analyzer (Model 600A, CH Instrument, USA) to determine the redox potential values for **1**, **2**, the amino derivatives (**3a–e**, **4** and **5**) and menadione (Sigma Chemicals, USA). A three-electrode assembly consisting of platinum wires (working and auxiliary electrodes) and Ag–AgCl (reference electrode) was used for the measurements. Solutions of the test compound (1 mM) were prepared in *N*,*N*-dimethylformamide (DMF) containing tetrabutyl ammonium perchlorate (TBAP; 0.1 M; Fluka Chemie AG, Buchs, Switzerland) as the supporting electrolyte. Each solution was purged with nitrogen for 5 min prior to a measurement, and the cyclic voltammograms were recorded in the range of 0.0 to −1.2 V at a scan rate of 100 mV/s under nitrogen atmosphere [39].

### 4.2. Biological studies

#### 4.2.1. Antitumor assay in vivo

Closed colony-bred Swiss A mice were obtained from Chittaranjan National Cancer Institute, Calcutta, and housed in polypropylene cages under standard laboratory conditions. They were given standard food pellets (Lipton, India; 5 g per mouse), water *ad libitum*, and were treated according to the guidelines of the Institutional Animal Ethics Committee. EAC cells, obtained from CNCI, Calcutta, were serially maintained by routine intraperitoneal (ip) transplantation of $1 \times 10^6$ cells/mouse in female Swiss A mice (18–20 g). The cells were harvested in phosphate-buffered saline (PBS; pH 7.4) on day 12–14 post-transplantation, and inoculated ip in groups of female Swiss A mice ($1 \times 10^5$ cells/mouse) on day '0' [14,29]. The test compound, dissolved in DMSO was administered ip (0.1 mL; 1 mg $kg^{-1}$ $day^{-1}$; five doses) to the 'treated' group (10 mice) on alternate days, starting from day '1'. The tumor growth was monitored daily by recording the survival and the individual body weight of each mouse. One set of experimental mice was sacrificed for collecting the blood serum and liver on day '16'. Biochemical assay of liver function enzymes, viz. lactate dehydrogenase (LDH) and alkaline phosphatase (AP), was carried out on the blood serum, and the liver tissues were subjected to histopathological examination in order to assess the alterations in these parameters of the 'treated' groups with respect to the tumor-bearing 'control' as well as 'normal' uninfected mice [14]. All the above experiments were repeated at least two to three times.

#### 4.2.2. Antitumor assay in vitro

*4.2.2.1. Cell culture.* Human cancer cell lines (A375 and Hep2), obtained from National Centre for Cell Science, Pune, India, were grown in DMEM (GIBCO-BRL, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated FCS (GIBCO-BRL, Gaithersburg, MD, USA) containing 5% mixture of penicillin (100 U/mL), streptomycin (100 μg/mL) and gentamicin (3 μg/mL) in the presence of 5% $CO_2$ in air at 37 °C and routinely subcultured using a 0.25% trypsin–0.02% EDTA solution.

EAC cells, obtained from Chittaranjan National Cancer Institute, Calcutta, were serially maintained in female Swiss A mice (6–8 weeks old; 18–20 g) by routine intraperitoneal (ip) transplantation. For this experiment, cells were collected on day 12–14 post-transplantation, suspended in phosphate-buffered saline (PBS; pH 7.4), centrifuged and washed with cold PBS. The pellet was resuspended in the RPMI 1640 medium without phenol red (Sigma Chemical Company, USA), supplemented with serum and antibiotics as above for incubation to be carried out for the desired experiment in a humidified atmosphere of 5% $CO_2$ in air at 37 °C.

Fresh heparinized whole blood was collected from normal human volunteer with informed consent. PBMCs were isolated by Ficoll–Paque density gradient centrifugation [40]. The blood (5 mL) was layered carefully over the Hypaque (3 mL, Sigma Diagnostics, USA) and centrifuged at room temperature at 1000 rpm for 45 min. The buffy coat layer containing PBMC at the interface was carefully taken out, washed twice with PBS and centrifuged at 1500–2000 rpm for 10 min. The cells were suspended in RPMI 1640 with phenol red (GIBCO-BRL, Gaithersburg, MD, USA), supplemented with 20% FCS and antibiotics (as above), and incubated in the presence of 5% $CO_2$ in air at 37 °C.

*4.2.2.2. Assessment of cytotoxicity.* In vitro growth inhibition effect of the test compounds on A375, Hep2, EAC and PBMC was assessed by colorimetric determination of the conversion of MTT (Sigma Chemicals, USA) into 'formazan blue' by the

living cells [32]. Briefly, cells ($2 \times 10^5$/mL) were seeded in 96-well flat-bottomed microplates (Nunc, Roskilide, Denmark), and treated with different concentrations, in triplicates, of the test compounds appropriately diluted with DMSO. After 24 h incubation at 37 °C in a humidified atmosphere of 5% $CO_2$, the medium was replaced with MTT solution (100 μL, 1 mg/mL in sterile PBS) for further 24 h incubation (4 h in the case of EAC cells). The supernatant was aspirated carefully, the precipitated crystals of 'formazan blue' were solubilized by adding DMSO (200 μL) to each well, and the optical density was measured with a microplate reader (BIO-RAD, Model 680, Microplate Reader) at a wavelength of 570 nm. Doxorubicin (Sigma Chemicals, USA) was used as the positive control in this experiment. The result represents the mean of three independent experiments and is expressed as $IC_{50}$, the concentration at which the optical density of the treated cells was reduced by 50% with respect to the untreated control.

*4.2.2.3. Evaluation of ROS generation in EAC cells.* The production of intracellular ROS in EAC cells was assessed with the oxidation sensitive, lipid permeable fluorescent probe DCFH-DA (Sigma Chemicals, USA), which readily diffuses into the cells and gets hydrolysed by intracellular esterase to form DCFH [36]. This is trapped within the cells and oxidized by cellular hydrogen peroxide or other oxidizing ROS to produce highly fluorescent compound DCF. EAC cells ($2 \times 10^5$ cells/mL) were first loaded with DCFH-DA (10 μM) at 37 °C for 20 min followed by treatment with diospyrin and its derivatives (0.4 μM) for 1 h. The increase in fluorescence intensity of DCF, as a measure of ROS, was determined by a spectrofluorimeter (Shimadzu RF-5000) at $\lambda_{ex}/\lambda_{em} = 490/520$ nm (slit width, 5 nm). The same determination was repeated in the presence of NAC (Sigma Chemicals, USA), an antioxidant, by pre-incubating the cells with NAC (100 μM) for 2 h, followed by treatment with DCFH-DA and incubated with the sample as done before [34]. A minimum of three separate determinations were carried out for each compound.

### 4.3. Statistical analysis

Statistical analysis to evaluate the significance of any differences between the data of two chosen groups was done by using Student's *t*-test. The $IC_{50}$ values were calculated by using linear regression analysis (MINITAB Release 13.31, USA).

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## References

[1] G. Powis, Free Radical Biol. Med. 6 (1989) 63–101.
[2] C. Asche, Mini Rev. Med. Chem. 5 (2005) 449–467.
[3] P.J. O'Brien, Chem. Biol. Interact. 80 (1991) 1–41.
[4] F. Fariña, M. Martinez-Utrilla, V. Stefani, Synthesis (1985) 781–784.
[5] E.A. Couladouros, Z.F. Plyta, S.A. Haroutounian, V.P. Papageorgiou, J. Org. Chem. 62 (1997) 6–10.
[6] G. Zagotto, R. Supino, E. Favini, S. Moro, M. Palumbo, Il Farmaco 55 (2000) 1–5.
[7] S. Bittner, S. Gorohovsky, O. Paz-Tal (Levi), J.Y. Becker, Amino Acids 22 (2002) 71–93.
[8] L. Teich, K.S. Daub, V. Krügel, L. Nissler, R. Gebhardt, K. Eger, Bioorg. Med. Chem. 12 (2004) 5961–5971.
[9] N.P. Mischenko, S.A. Fedoreyev, N.D. Pokhilo, V.Ph. Anufriev, V.A. Denisenko, V.P. Glazunov, J. Nat. Prod. 68 (2005) 1390–1393.
[10] S.C. Kuo, T. Ibuka, L.J. Huang, J.C. Lien, S.R. Yean, S.C. Huang, D. Lednicer, S. Morris-Natschke, K.H. Lee, J. Med. Chem. 39 (1996) 1447–1451.
[11] M.-E. Suh, S.-Y. Park, H.-J. Lee, Bull. Korean Chem. Soc. 23 (2002) 417–422.
[12] H.-S. Huang, H.-F. Chiu, W.-C. Lu, C.-L. Yuan, Chem. Pharm. Bull. 53 (2005) 1136–1139.
[13] B. Hazra, P. Sur, D.K. Roy, A. Banerjee, Planta Med. 51 (1984) 295–297.
[14] B. Hazra, B. Kumar, S. Biswas, B.N. Pandey, K.P. Mishra, Toxicol. Lett. 157 (2005) 109–117.
[15] S. Pal, B. Hazra, A. Banerjee, R. Ray, D.K. Bhattacharya, Phytother. Res. 10 (1996) 393–397.
[16] S. Chakraborty, M. Roy, B. Hazra, R.K. Bhattacharya, Cancer Lett. 188 (2002) 85–93.
[17] B. Hazra, M. Das Sarma, B. Kumar, S. Basu, K. Das, B.N. Pandey, K.P. Mishra, Chemotherapy 53 (2007) 173–176.
[18] B. Kumar, J. Joshi, A. Kumar, B.N. Pandey, B. Hazra, K.P. Mishra, Mol. Cell. Biochem. 304 (2007) 287–296.
[19] M. Das Sarma, R. Ghosh, A. Patra, B. Hazra, Bioorg. Med. Chem. 15 (2007) 3672–3677.
[20] H. Nohl, W. Jordan, R.J. Youngman, Adv. Free Radical Biol. Med. 2 (1986) 211–279.
[21] D. Barasch, O. Zipori, I. Ringel, I. Ginsberg, A. Samuni, J. Katzhendler, Eur. J. Med. Chem. 34 (1999) 597–615.
[22] F.C. de Abreu, P.A.L. Ferraz, M.O.F. Goulart, J. Braz. Chem. Soc. 13 (2002) 19–35.
[23] U. Sanyal, S. Bhattacharyya, A. Patra, B. Hazra, J. Chromatogr. A 1017 (2003) 225–232.
[24] M. Yoshida, K. Mori, Eur. J. Org. Chem. (2000) 1313–1317.
[25] W.T.A. Harrison, O.C. Musgrave, Acta Crystallogr. C 60 (2004) 399–401.
[26] S.G. Renou, S.E. Asis, M.I. Abasolo, D.G. Bekerman, A.M. Bruno, Pharmazie 58 (2003) 690–695.
[27] S. Bittner, D. Lempert, Synthesis (1994) 917–919.
[28] A. Ashnagar, J.M. Bruce, P.L. Dutton, Biochim. Biophys. Acta 801 (1984) 351–359.
[29] R.I. Geran, N.H. Greenberg, M.M. Macdonald, A.M. Schumacher, B.J. Abbott, Cancer Chemother. Rep. 3 (1972) 1–103.
[30] C.M. Woolf, in: D. Van (Ed.), Principles of Biometry, Norstrand Company Inc., New York, London, 1986, pp. 290–296.
[31] M. Ghosh, U. Sadhu, S. Bhattacharya, S. Dutta, B. Bhattacharya, U. Sanyal, Cancer Lett. 138 (1999) 107–114.

[32] T. Mosmann, J. Immunol. Methods 65 (1983) 55–63.
[33] M. Das Sarma, R. Ghosh, A. Patra, R. Chowdhury, K. Chaudhuri, B. Hazra, Org. Biomol. Chem. 5 (2007) 3115–3125.
[34] B. Kumar, Antitumor activity of diospyrin, a naphthoquinonoid compound and its derivatives, evaluated individually, as well as in combination with radiation treatment, Ph.D. Dissertation, Jadavpur University, 2006.
[35] J.S. Driscoll, G.F. Hazard Jr., H.B. Wood Jr., A. Goldin, Cancer Chemother. Rep. 4 (1974) 1–362.
[36] F. Gao, J. Yi, J.Q. Yuan, G.Y. Shi, X.M. Tang, Cell Res. 14 (2004) 81–85.
[37] J. Chandra, A. Samali, S. Orrenius, Free Radical Biol. Med. 29 (2000) 323–333.
[38] K.R. Kunz, B.S. Iyengar, R.T. Dorr, D.S. Alberts, W.A. Remers, J. Med. Chem. 34 (1991) 2281–2286.
[39] J. Johnson Inbaraj, R. Gandhidasan, R. Murugesan, Free Radical Biol. Med. 26 (1999) 1072–1078.
[40] X. Chen, M. Lowe, T. Herliczek, M.J. Hall, C. Danes, D.A. Lawrence, K. Keyomarsi, J. Natl. Cancer Inst. 92 (2000) 1999–2008.