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Original article

Synthesis and cytotoxicity evaluation of some benzimidazole-4,7-diones as bioreductive anticancer agents

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Dedicated to Professor José Maldonado for his contribution to the Science of Pharmaceutical Organic Chemistry.

Abstract

New benzimidazole-4,7-diones substituted at 2-position were synthesized via a microwave-assisted reaction using 2-chloromethyl-1,5,6-trimethyl-1*H*-benzimidazole-4,7-dione **5b** as a key intermediate compound. Their cytotoxicity has been evaluated on colon, breast and lung cancer cell lines. The dimer **17** was shown to possess excellent cytotoxicity comparable to that of mitomycin C.

Keywords: Benzimidazole-4,7-dione; Mitomycin C; Antitumor; Microwave

1. Introduction

Mitomycin C (Fig. 1) is an antitumor drug discovered in the 1950s by Japanese microbiologists. Several close structural variants of mitomycin C have since been isolated, collectively called as mitomycins [1]. Mitomycin C has a variety of specific biological effects in mammalian cells, including selective inhibition of DNA synthesis, mutagenesis, and increase of genetic recombination, chromosome breakage and sister chromatid exchange [2]. Many solid tumors possess viable hypoxic cells that are resistant to radiation therapy and some anticancer chemotherapy protocols. Strategies designed to overcome this radioresistance include the use of bioreductive drugs, which exhibit selective toxicity towards hypoxic cells, both in vitro and in vivo [3]. Mitomycins and the corresponding mitosenes are well-known examples of reductive alkylating quinones [4]. Bioreduction of such quinones is mediated through one- or two-electron reduction by different cellular enzyme complexes such as cytochrome P450 oxidoreductase, cytochrome b5 oxidoreductase and DT-diaphorase. The DT-diaphorase is an NADPH-dependent enzyme that reduces quinones and other substrates into the corresponding two-electron reduction products [5]. These reductive alkylating quinone methide species, which are formed upon reduction of leaving groups, are highly reactive and produce cytotoxic effects by reacting with DNA of tumor cells [6]. Among these agents, the pyrrolo[1,2-*a*]benzimidazoles (PBIs) were found to possess cytotoxicity with minimal antitumor activity [7]. The indoloquinone EO9 was considered to be a promising antitumor agent, but phase I clinical trials revealed short plasma half-lives as well as toxicity [8].

Biological properties also have been observed in some benzimidazolediones [9], and 5-amino-6-bromobenzimidazole-4,7-dione showed good antitumor activity in vivo on P388 leukemia cell line [10]. In most cases the activity is related to the ability of quinone to accept one or two electrons, forming reactive cytotoxic species [11]. The substituents on the quinone ring play a significant role in determining the biological properties. The

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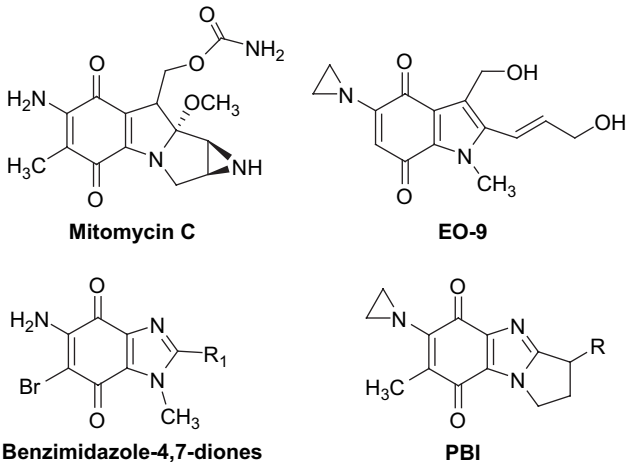


Fig. 1. Structures of azamitosene, benzimidazole-4,7-dione, and indoloquinone derivatives and of mitomycin C.

presence of electron-withdrawing or electron-donating groups, by varying the quinone redox properties, influences its capacity to interfere with DNA synthesis [12]. Moreover, studies on arylsulfone containing antitumor agents showed that substituents of the sulfone group play an important role in the biological activity [13–16].

Concurrently, the beneficial effects of microwave irradiation are finding an increased role in process chemistry, especially when conventional methods require forcing conditions or prolonged reaction times [17–19]. The possibilities offered by this technology are particularly attractive for multi-step synthesis [20–22] and drug discovery process, where high yielding protocols and avoidance or ease of purification are highly desirable.

On the basis of the above considerations, we have synthesized a series of variously substituted benzimidazole-4,7-diones and tested them for their antitumor activity. Substituents were selected with different electronic and solubility characteristics, while others were chosen for their alkylating properties, with the aim of investigating the substituent effects at the 2-, 5- and 6-position.

2. Chemistry

We applied Day's method [23] to prepare (4,7-dimethoxy-5,6-dimethyl-1*H*-benzimidazol-2-yl)-methanol **2b** with significant modifications (Scheme 1) [24,25]. We present herein a synthetic method based on a six-step synthesis (overall yield 50%), which had been previously developed in our laboratory [26], for the preparation of 2-(chloromethyl)-4,7-dimethoxy-1-methyl-1*H*-benzimidazole derivatives.

The 2-chloromethyl-1,5,6-trimethyl-1*H*-benzimidazole-4,7-dione **5b** was obtained after an oxidative demethylation of **4b** by treatment with cerium ammonium nitrate (CAN), as shown in Scheme 2.

The dihydroxy compound **6** was obtained by demethylation of **4a**, using BBr_3/CH_2Cl_2 at −78 °C. Oxidation of **6** using potassium dichromate in water at room temperature gave **5a** in 59% overall yield (Scheme 3).

The bromo derivative **8** was obtained in 58% yield from **4a** via refluxing in HBr (48%) followed by oxidation with $K_2Cr_2O_7$ (Scheme 4).

The sulfonyl derivatives **9** and **10** were prepared under microwave irradiation (H_2O, 4 min), by treatment of **5b** with the sodium salts of the corresponding sulfinic acid in aqueous solution, according to a previously described method (Scheme 5) [26]. Reaction of **5b** with an excess of nitronate anions, under microwave irradiation, afforded the corresponding benzimidazole-4,7-diones **11–13**, in 73–85% yield via an $S_{RN}1$ mechanism (Scheme 5). The malonate anions gave, under microwave irradiation, the corresponding diesters **14** and **15**, in 67 and 72% yield, respectively.

Treatment of **11** with TBAOH resulted in the elimination of HNO_2 with the formation of the alkene **16** in 78% yield (Scheme 6).

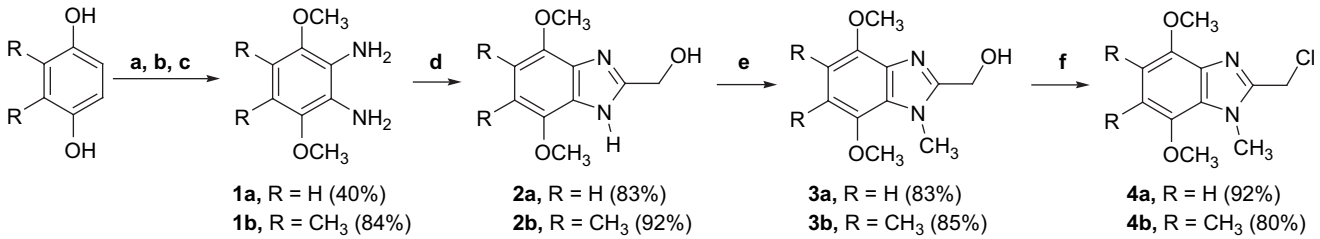
According to a study recently carried out at our laboratory on bis-alkylating derivatives [27], we sought thereafter to prepare a new bioreductive bis-alkylating agent in quinone series, aiming at comparing the cytotoxic activity of the quinone derivatives comprising various substituents in 2-position with that of a dimer presenting two reducible chloromethyl groups.

Thus, oxidation of *para*-dimethoxybenzene derivatives by CAN generally leads to the corresponding quinones. There are, however, some unsubstituted *para*-dimethoxybenzene derivatives, for which an oxidative coupling is observed, leading to a dimer [28]. Indeed, the oxidative demethylation of **4a**, by treatment with CAN, gave the dimer **17** in 38% yield (Scheme 7).

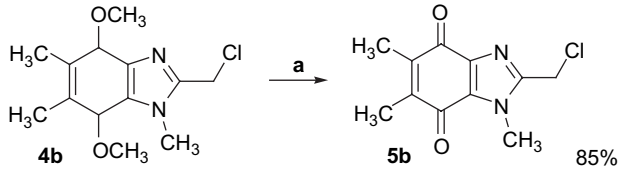
The exact structure of this compound was determined according to HMBC experiment.

3. Biological studies

Evaluation of cytotoxicity of the synthesized compounds was performed on different cancer cell lines using MTT assay.



Scheme 1. Reagents and conditions: (a) KOH, $(CH_3)_2SO_4$, CH_3OH, reflux, 1 h; (b) R = H: HNO_3 62%, 1 h at 0 °C, 1 h at rt, 1 h at 100 °C or R = CH_3: $(CH_3CO)_2O$, HNO_3 fuming, 90 °C, 1 h; (c) Sn, HCl; (d) $HOCH_2CO_2H$, 4 N HCl, microwave, 800 W, 1 h 30 min; (e) $LiN(TMS)_2$, THF, 20 min, then CH_3I, 7 h; (f) $SOCl_2$, $CHCl_3$, reflux, 4 h.



Scheme 2. Reagents and conditions: (a) $Ce(NH_4)_2(NO_3)_6$, CH_3CN, rt, 3 h.

A first screening on breast (T47D), lung (A549) and colon (HT29) cancer cell lines using 10^{-5} and 10^{-4} M of each compound was done. Compounds which do not exhibit inhibition of cell viability higher than 50% at 10^{-4} M are not further characterized and their IC_{50} value are reported as $>10^{-4}$ M. Compounds exhibiting inhibition of cell viability higher than 50% at 10^{-4} M have been further evaluated using the Chou and Talalay method [29] to determine the IC_{50} values (see Section 5). Summary of the results obtained is presented in Table 1.

Compound **17** is far more effective in all cell lines tested with an efficient IC_{50} close to 3 μM. Other compounds exhibit variable results depending on the cell line tested. The other compounds are arranged in an order of decreasing activity: **17** ≫ **9** = **10** > **11** > **5b** for the mammary T47D cell line; however, **17** ≫ **11** > **9** > **5b** for the lung A549 cell line and **17** ≫ **5b** = **11** for the colon HT29 cell line.

Apart from the diquinone **17**, the cytotoxic activity of other tested quinones are shown by those bearing a methyl group in positions 5 and 6, and a chloromethyl or a substituted sulfonyl group in position 2 (compounds **5b**, **9**, **10**, and **17**). The quinones having no substituents at positions 5 and 6 (compound **5a** and **8**) showed lower activity on the different cellular lines tested.

Besides the changes in structure, which explain the differential effects of these compounds, variable effects observed depending on the different cell lines that might result from a different metabolic background. Indeed these benzimidazole-4,7-dione derivatives, as the prototypic molecule mitomycin C, are known to be bioactivated through one- or two-electron reduction [30]. One important mechanism for two-electron reduction occurs through DT-diaphorase (NADPH: quinone oxidoreductase 1; NQO1), which is significantly active in HT29 cell [31]. Compound **17** exhibits the lowest IC_{50} concentration whatever the cell line considered compared to other compounds. This derivative **17** with two chloride nucleofuges, corresponding to a bioreductive bis-alkylating agent susceptible to undergo two monoelectronic transfers [32], exhibits a cytotoxic activity higher than the ones of the derivatives presenting one chloride nucleofuge. The previous biological data suggest that compound **17** would present a different profile of DT-diaphorase bioactivation compared to mitomycin C. Compound **17** exhibits a similar dose effect to mitomycin C (Fig. 2). Inhibition of DT-diaphorase by 2 μM of dicoumarol significantly increases the IC_{50} value of mitomycin C from 0.9 ± 0.1 to 2.1 ± 0.2. In contrast, IC_{50} of compound **17** is unchanged by addition of dicoumarol 2.8 ± 0.2 versus 2.7 ± 0.2.

For derivative **17**, the results show that the activity of the compound is insensitive to DT-diaphorase activity modulation, which would tend to show that the mechanism of action of the dimer **17** passes primarily by a mechanism of monoelectronic bioreduction. These results suggest the possibility to obtain new bioreductive alkylating agents with limited resistance compared to mitomycin C.

4. Conclusion

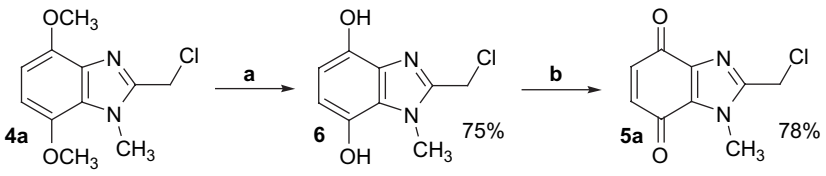
The microwave-assisted reaction gave new benzimidazole-4,7-diones from the intermediate 2-chloromethyl-1,5,6-trimethyl-1*H*-benzimidazole-4,7-dione **5b**, which was prepared in a high overall yields (50%) in seven steps. The compounds thus obtained were tested against different cancerous mammalian cell lines to evaluate their dose–response relationships.

Among all the tested compounds, the dimer **17** proved to be most effective on breast (T47D), lung (A549) and colon (HT29) cancer cells. This efficiency is comparable to that of the reference prototype bioreductive anticancer drug mitomycin C. It is well-known that this drug requires metabolism conversion to produce reactive intermediates which irreversibly binds to DNA. Mitomycin C reduction, through two-electron addition mechanism, is catalyzed by various enzyme complexes and in particular by DT-diaphorase. The limitation of some bioreductions is a factor of resistance to mitomycin C. Based on our in vitro results, compound **17**, insensitive to DT-diaphorase bioreduction, may participate in the development of mitomycin C-like agents, presenting lower tumoral cell resistance.

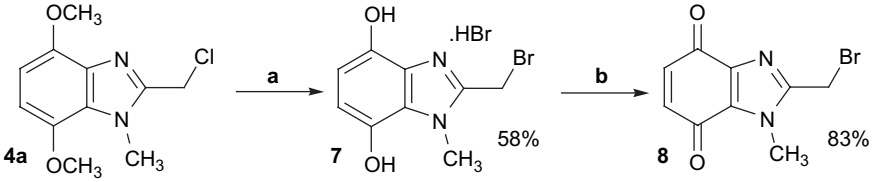
5. Experimental protocols

5.1. Chemistry

Melting points were determined on Büchi B-540 and are uncorrected. Elemental analyses were performed by the Microanalyses center of the University of Aix-Marseille 3 and of the INP-ENSCT (Toulouse, France). Both ^1H and ^{13}C NMR spectra were determined on a Bruker ARX 200 spectrometer. The ^1H chemical shifts are reported as ppm downfield from tetramethylsilane (Me_4Si), and the ^{13}C chemical shifts were referenced to the solvent peak: $CDCl_3$ (76.9 ppm). Solvents were



Scheme 3. Reagents and conditions: (a) BBr_3, CH_2Cl_2, −78 °C, 3 h; (b) $K_2Cr_2O_7$, H_2O, rt, 2 h.



Scheme 4. Reagents and conditions: (a) HBr (48%), reflux, 3 h; (b) $K_2Cr_2O_7$, H_2O, rt, 2 h.

dried by conventional methods. The following adsorbent was used for column chromatography: silica gel 60 (Merck, particle size 0.063–0.200 mm, 70–230 mesh ASTM). TLC was performed on 5 cm × 10 cm aluminum plates coated with silica gel 60F-254 (Merck) in an appropriate solvent.

Multimode reactor: ETHOS Synth Lab station (Ethos start, Milestone Inc.). The multimode microwave has a twin magnetron (2 × 800 W, 2.45 GHz) with a maximum delivered power of 1000 W in 10 W increments (pulse irradiation). Built-in magnetic stirring (Teflon-coated stirring bar) was used in all operations. During experiments, time, temperature and power were measured with the ''easy WAVE'' software package. The temperature was measured throughout the reaction and evaluated by an infrared detector, which indicated the surface temperature. Analysis indicated by the symbols of the elements, were within ±0.4% of the theoretical values (see supplementary data).

The derivatives **1b–5b**, **9**, **10** [26], **1a–4a**, **6** and **7** [33] were prepared as previously described.

5.1.1. 2-Chloromethyl-1-methyl-1H-benzimidazole-4, 7-dione *5a*

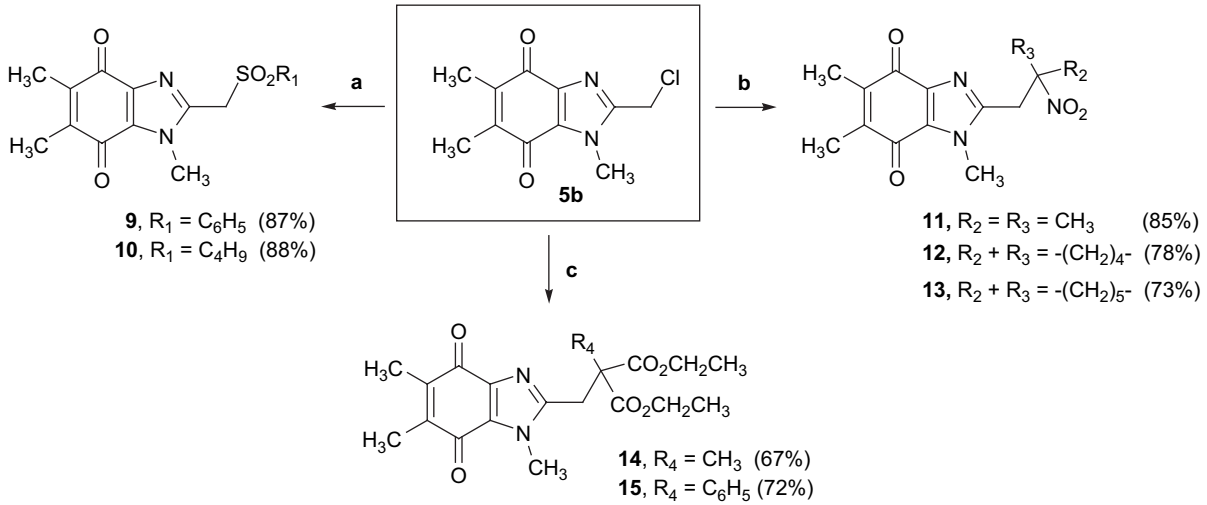
To a solution of potassium dichromate (13.8 g, 46.9 mmol) in water (50 mL), 2-(chloromethyl)-1-methyl-1*H*-benzimidazole-4,7-diol **6** (2 g, 9.4 mmol) was added portionwise. The reaction mixture was stirred for 2 h and extracted with chloroform (3 × 50 mL). The combined organic layer was dried over magnesium sulfate and evaporated under reduced pressure. Purification of the crude product on silica gel by eluting with chloroform–ethanol (9/1) and recrystallization from propan-2-ol gave **5a** (78%) as a yellow solid; mp 164–166 °C; ^{1}H NMR (200 MHz, DMSO-d_6) δ 3.95 (3H, s, NCH_3), 5.03 (2H, s, CH_2), 6.74 (2H, d, $J = 8$ Hz, CH); ^{13}C NMR (50 MHz, DMSO-d_6) δ 32.36 (CH_2), 35.93 (NCH_3), 131.79 (C), 136.57 (2CH), 140.05 (C), 149.97 (C), 178.54 (C), 180.83 (C); Anal. $C_9H_7ClN_2O_2$ (C, H, N).

5.1.2. 2-(Bromomethyl)-1-methyl-1H-benzimidazole-4, 7-dione *8*

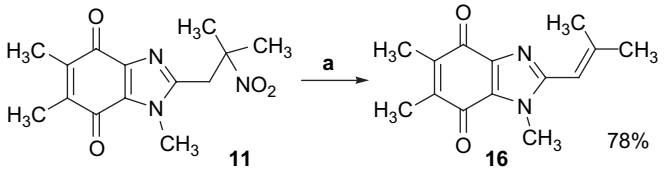
The above-mentioned procedure reported for **5a** was followed using 2-(bromomethyl)-1-methyl-1*H*-benzimidazole-4,7-diol hydrobromide **7** to give **8** (83%) as a white solid; mp 154–156 °C [34]; ^{1}H NMR (200 MHz, $CDCl_3$) δ 4.03 (3H, s, NCH_3), 4.57 (2H, s, CH_2), 6.66 (2H, d, $J = 8$ Hz, CH); ^{13}C NMR (50 MHz, $CDCl_3$) δ 20.04 (CH_2), 32.53 (NCH_3), 131.54 (C), 136.27 (CH), 136.55 (CH), 140.82 (C), 149.79 (C), 178.37 (C), 180.41 (C); Anal. $C_9H_7BrN_2O_2$ (C, H, N).

5.1.3. 1,5,6-Trimethyl-2-(2-methyl-2-nitropropyl)-1H-benzimidazole-4,7-dione *11*

To a solution of 2-nitropropane lithium salt (0.35 g, 3.7 mmol) in H_2O (3 mL), 2-chloromethyl-1,5,6-trimethyl-1*H*-benzimidazole-4,7-dione **5b** (0.3 g, 1.26 mmol) was added dropwise. The reaction mixture was irradiated in a microwave oven (Ethos start) for 10 min at 100 °C, with a power of 800 W. The crude product was dissolved in dichloromethane (20 mL). The organic layer was then dried over anhydrous magnesium sulfate, and evaporated under reduced pressure. Purification by column



Scheme 5. Reagents and conditions: (a) R-$SO_2^-Na^+$, H_2O, microwave, 800 W, 80 °C, 4 min; (b) $N(Bu)_4^+$ $^-CR_1R_2NO_2$, H_2O, microwave, 800 W, 10 min, 100 °C; (c) $N(Bu)_4^+$ $^-C(CO_2CH_2CH_3)_2$, H_2O, microwave, 800 W, 10 min, 100 °C.



Scheme 6. Reagents and conditions: (a) CH_2Cl_2, TBAOH 40%, 3 h, rt.

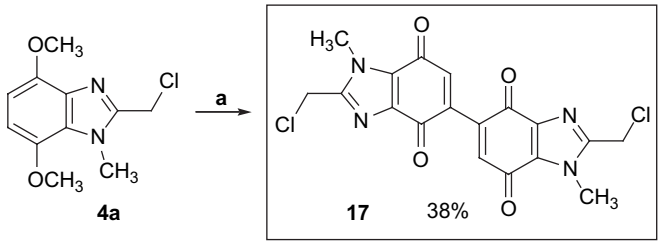
chromatography on silica gel using chloroform–ethyl acetate (7/3) as eluent and recrystallization from propan-2-ol gave **11** (85%) as a yellow solid; mp 183–184 °C; ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.78 (6H, s, $2CH_3$), 2.06 (3H, s, CH_3), 2.09 (3H, s, CH_3), 3.39 (2H, s, CH_2), 3.92 (3H, s, NCH_3); ^{13}C NMR (50 MHz, $CDCl_3$) δ 12.08 (CH_3), 12.41 (CH_3), 26.37 ($2CH_3$), 32.29 (NCH_3), 36.31 (CH_2), 87.11 (C), 130.77 (C), 139.95 (C), 140.69 (C), 141.08 (C), 149.19 (C), 178.76 (C), 180.96 (C); Anal. $C_{14}H_{17}N_3O_4$ (C, H, N).

5.1.4. 1,5,6-Trimethyl-1H-benzimidazole-4,7-dione derivatives *12–15*

To a solution of 40% tetrabutylammonium hydroxide in water (3.7 mmol) and corresponding nitroalkane or malonate derivative (3.7 mmol), 2-chloromethyl-1,5,6-trimethyl-1*H*-benzimidazole-4,7-dione **5b** (0.3 g, 1.26 mmol) was added. The reaction mixture was irradiated in a microwave oven (Ethos start) for 10 min at 100 °C, with a power of 800 W. The crude product was dissolved in dichloromethane (20 mL). The organic layer was then dried over anhydrous magnesium sulfate, and evaporated under reduced pressure. Purification by column chromatography on silica gel using chloroform–ethyl acetate (7/3) as eluent and recrystallization from propan-2-ol gave corresponding products **12** and **13** (78 and 73%) or **14** and **15** (67 and 72%).

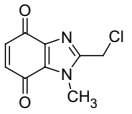
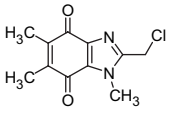
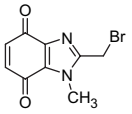
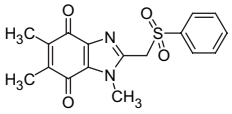
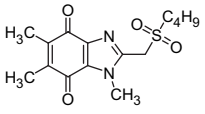
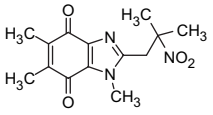
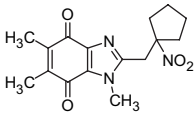
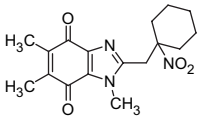
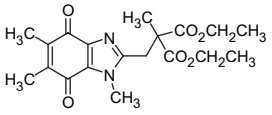
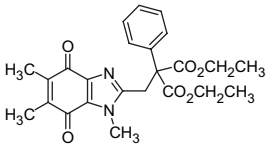
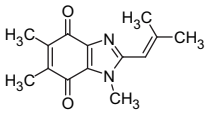
*5.1.4.1. 1,5,6-Trimethyl-2-[(1-nitrocyclopentyl)methyl]-1H-benzimidazole-4,7-dione **12***. Yellow solid; mp 162–163 °C; ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.78–1.84 (4H, m, $2CH_2$), 2.04 (3H, s, CH_3), 2.08 (3H, s, CH_3), 2.19–2.63 (4H, m, $2CH_2$), 3.46 (2H, s, CH_2), 3.90 (3H, s, NCH_3); ^{13}C NMR (50 MHz, $CDCl_3$) δ 12.06 (CH_3), 12.39 (CH_3), 24.25 ($2CH_2$), 32.16 (NCH_3), 34.86 (CH_2), 37.73 ($2CH_2$), 97.73 (C), 130.75 (C), 139.91 (C), 140.66 (C), 141.11 (C), 149.70 (C), 178.78 (C), 180.99 (C); Anal. $C_{16}H_{19}N_3O_4$ (C, H, N).

*5.1.4.2. 1,5,6-Trimethyl-2-[(1-nitrocyclohexyl)methyl]-1H-benzimidazole-4,7-dione **13***. Yellow solid; mp 183–184 °C; ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.78–1.84 (6H, m, $3CH_2$),



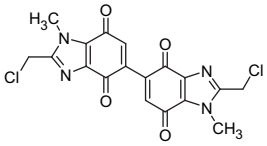
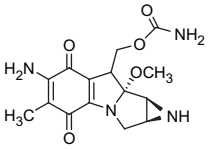
Scheme 7. Reagents and conditions: (a) $Ce(NH_4)_2(NO_3)_6$, CH_3CN, rt, 2 h.

Table 1
Cytotoxicity IC_{50} values in μM for the tested compounds against three cancer cell lines

Compound	Formula	T47D[a] (breast)	A549[a] (lung)	HT29[a] (colon)
5a		>100	>100	>100
5b		100 ± 8	**40 ± 5**	**15 ± 4**
8		>100	>100	>100
9		**10 ± 3**	**8 ± 2**	>100
10		**10 ± 4**	**10 ± 3**	>100
11		>100	>100	>100
12		>100	>100	>100
13		>100	>100	>100
14		>100	>100	>100
15		>100	>100	>100
16		>100	>100	>100

(*continued on next page*)

Table 1 (*continued*)

Compound	Formula	T47D[a] (breast)	A549[a] (lung)	HT29[a] (colon)
17		**3 ± 1**	**3 ± 1**	**3 ± 1**
MC		–	–	**0.9 ± 0.1**

[a] Results express the mean $IC_{50} \pm SEM$ obtained from three independent experiments.

2.04 (3H, s, CH_3), 2.06 (3H, s, CH_3), 2.16–2.25 (4H, m, $2CH_2$), 3.46 (2H, s, CH_2), 3.90 (3H, s, NCH_3); ^{13}C NMR (50 MHz, $CDCl_3$) δ 12.04 (CH_3), 12.40 (CH_3), 24.20 ($2CH_2$), 25.60 (CH_2), 32.33 (NCH_3), 34.79 (CH_2), 37.73 ($2CH_2$), 97.65 (C), 130.68 (C), 139.85 (C), 140.60 (C), 141.01 (C), 149.60 (C), 178.88 (C), 180.90 (C); Anal. $C_{17}H_{21}N_3O_4$ (C, H, N).

5.1.4.3. Diethyl 2-methyl-2-[(1,5,6-trimethyl-4,7-dioxo-4,7-dihydro-1H-benzimidazol-2-yl)methyl]malonate ***14***. Yellow oil; ^1H NMR (200 MHz, $CDCl_3$) δ 1.24 (6H, t, $J = 7.2$ Hz, $2CH_3$), 1.70 (3H, s, CH_3), 2.06 (3H, s, CH_3), 2.09 (3H, s, CH_3), 3.48 (2H, s, CH_2), 3.84 (3H, s, NCH_3), 4.20 (4H, q, $J = 7.2$ Hz, $2CH_2$); ^{13}C NMR (50 MHz, $CDCl_3$) δ 12.36 (CH_3), 12.40 (CH_3), 13.96 ($2CH_3$), 20.56 (CH_3), 32.50 (NCH_3), 32.76 (CH_2), 61.91 ($2CH_2$), 140.06 (C), 140.50 (C), 141.02 (C), 148.10 (C), 169.27 (C), 169.48 (C), 178.81 (2C), 180.59 (2C); Anal. $C_{19}H_{24}N_2O_6$ (C, H, N).

5.1.4.4. Diethyl 2-phenyl-2-[(1,5,6-trimethyl-4,7-dioxo-4,7-dihydro-1H-benzimidazol-2-yl)methyl]malonate ***15***. Yellow oil; ^1H NMR (200 MHz, $CDCl_3$) δ 1.15 (6H, t, $J = 7.0$ Hz, $2CH_3$), 2.05 (3H, s, CH_3), 2.08 (3H, s, CH_3), 3.60 (2H, s, CH_2), 3.85 (3H, s, NCH_3), 4.45 (4H, q, $J = 7.0$ Hz, $2CH_2$), 7.35 (5H, m, H ar); ^{13}C NMR (50 MHz, $CDCl_3$) δ 12.30 (CH_3), 12.33 (CH_3), 13.98 ($2CH_3$), 31.22 (NCH_3), 32.84 (CH_2), 60.89 ($2CH_2$), 126.85 (3CH), 127.43 (2CH), 140.11 (C), 140.12 (C), 141.21 (C), 141.35 (C), 148.14 (C), 169.32 (C), 169.53 (C), 178.87 (2C), 180.50 (2C); Anal. $C_{24}H_{26}N_2O_6$ (C, H, N).

5.1.5. 1,5,6-Trimethyl-2-(2-methylprop-1-enyl)-1H-benzimidazole-4,7-dione ***16***

To a solution of 1,5,6-trimethyl-2-(2-methyl-2-nitropropyl)-1*H*-benzimidazole-4,7-dione **11** (0.2 g, 0.68 mmol) in dichloromethane (30 mL) was added dropwise an aqueous solution of 40% tetrabutylammonium hydroxide (1.3 g, 0.68 mmol). The reaction mixture was stirred at room temperature for 3 h. The organic layer was then dried over anhydrous magnesium sulfate, and evaporated under reduced pressure. Purification by column chromatography on silica gel using chloroform–ethyl acetate (7/3) as eluent and recrystallization from propan-2-ol gave **16** (78%) as a red solid; mp 153–154 °C; ^1H NMR (200 MHz, $CDCl_3$) δ 2.00 (3H, s, CH_3), 2.05 (3H, s, CH_3), 2.09 (3H, s, CH_3), 2.22 (3H, s, CH_3), 3.89 (3H, s, NCH_3), 6.01 (1H, s, CH); ^{13}C NMR (50 MHz, $CDCl_3$) δ 12.11 (CH_3), 12.44 (CH_3), 20.98 (CH_3), 27.42 (CH_3), 31.84 (NCH_3), 109.53 (CH), 130.65 (C), 139.72 (C), 140.53 (C), 141.00 (C), 149.10 (C), 178.64 (C), 180.86 (C); Anal. $C_{14}H_{16}N_2O_2$ (C, H, N).

5.1.6. 2,2′-Bis(chloromethyl)-1,1′-dimethyl-5,5′-bi(1H-benzimidazole)-4,4′,7,7′-tetraone ***17***

To a solution of 2-(chloromethyl)-4,7-dimethoxy-1-methyl-1*H*-benzimidazole **4a** (5 g, 20.7 mmol) in acetonitrile (100 mL) was added dropwise a mixture of CAN (cerium ammonium nitrate) (32.6 g, 59.5 mmol) in water (40 mL). The reaction mixture was stirred for 3 h and the solvent was evaporated under vacuum. The residue was dissolved in chloroform and washed with water (3 × 30 mL). The organic layer was dried over magnesium sulfate and the solvent was removed under vacuum. Purification of the crude product on silica gel eluting with chloroform–ethyl acetate (9/1) and recrystallization from propan-2-ol gave **17** (38%) as a yellow solid; mp 221–222 °C; ^1H NMR (200 MHz, $CDCl_3$) δ 4.01 (3H, s, NCH_3), 4.59 (2H, s, CH_2), 6.50 (1H, s, CH); ^{13}C NMR (50 MHz, $CDCl_3$) δ 32.2

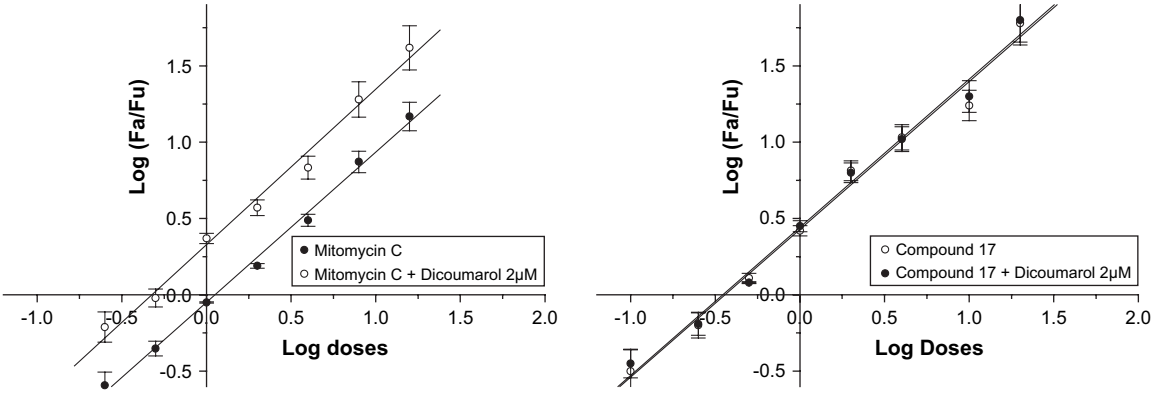


Fig. 2. Effect of DT-diaphorase inhibition on compound **17** efficiency compared to mitomycin C.

(NCH_3), 35.2 (CH_2), 131.6 (C), 135.7 (CH), 140.3 (C), 150.2 (C), 177.7 (C), 180.4 (C); Anal. $C_{18}H_{12}Cl_2N_4O_4$ (C, H, N).

5.2. Evaluation of cell cytotoxicity

5.2.1. Cell culture

Human lung (A549), breast (T47D) and colon (HT29) cancer cell lines were used to evaluate the impact on cell viability of each compound. A549 and T47D cells were cultured in RPMI 1640 medium and HT29 cells were maintained in Dulbecco's modified Eagle's medium (DMEM), all the mediums were supplemented with 10% fetal bovine serum (FBS) and 2 mM l-glutamine at 37 °C under a 5% CO_2 atmosphere. For each cell line, 70% confluent cell culture flask was trypsinized and cells were seeded in 96 well plates at a density of 3000 cells by well in the appropriate complete media; 24 h after seeding, the cells were treated with the different compounds or control vehicle solution (DMSO 0.1% in phosphate saline buffer); 48 h after treatment, viability was accessed by MTT assay.

5.2.2. Cytotoxicity assay

MTT assays determine the ability of viable cells to convert a soluble yellow tetrazolium salt (MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) into insoluble purple formazan crystals by the mitochondrial dehydrogenase enzymes. Cells were exposed to 0.5 mg/mL of MTT for 3 h at 37 °C in the appropriate complete medium. Medium and MTT were removed and after solubilization in dimethylsulfoxide (DMSO), the amount of insoluble formazan crystals was evaluated by measuring the optical density at 550 nm. Each condition was performed in triplicate. Each measurement was corrected from the optical density of MTT alone and expressed relative to the non-treated conditions. Determination of the concentration inhibiting 50% of cell viability (IC_{50}) was performed according to the methods of Chou and Talalay [29]. Briefly, the fraction of cell affected (F_a) and the fraction of cell unaffected (F_u) relative to 1 were determined from the viability assay. The log of (F_a/F_u) was plotted against the log of concentration for each compound. Log of IC_{50} was determined at the *y*-intercept. Standard error was evaluated through the 95% confidence interval. DT-diaphorase activity modulation was done by using dicoumarol in HT29 cells, which are known to exhibit high DT-diaphorase activity [31]. To inhibit DT-diaphorase activity, cells were incubated with 5 μM of dicoumarol for 30 min before treatment. Such dose of dicoumarol is reported to inhibit DT-diaphorase in HT29 and has been evaluated in our experimental condition to exclude a potential effect of dicoumarol (5 μM) alone on cell viability for 72 h.

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Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.ejmech.2007.11.020.

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