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Metronidazole twin ester prodrugs: synthesis, physicochemical properties, hydrolysis kinetics and antigiardial activity

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Abstract – A series of identical twin esters **3a–e** of metronidazole was synthesized and evaluated as potential prodrugs with improved physicochemical and pharmacokinetic properties. The synthesis of the twin esters **3a–e** was achieved by interaction of metronidazole with the respective dicarboxylic acid anhydride or their dichloride. Their structures were verified by elemental and spectroscopic analyses. The lipophilicity of metronidazole and the prodrugs **3a–e**, expressed as R_m values, were determined using reversed-phase TLC and revealed enhanced lipophilic properties compared with metronidazole. Reversion kinetics of the parent drug from its twin esters was investigated in aqueous buffer solutions (pH 1.2 and 7.4) as well as in biological media (80% human plasma and 20 % rat liver homogenate) at 37 °C using HPLC. In all cases, the hydrolysis followed pseudo-first-order kinetics in a two-step reaction (k_1 and k_2) via the intermediate formation of the respective metronidazole hemiester. All the synthesized twin ester prodrugs **3a–e** were proved to be chemically stable at acidic pH ($t_{1/2}$ ~ 25–72 h) and also at the physiological pH ($t_{1/2}$ ~ 13–40 h). Meanwhile, the release of the first molecule of metronidazole from its twin esters **3a–d** ensued rapidly in 80% human plasma ($t_{1/2}$ ~ 10–150 min) and in rat liver homogenate ($t_{1/2}$ ~ 4–55 min). The resulting hemiesters **2a–d** showed a sustained release of the second molecule in the same biological fluids ($t_{1/2}$ ~ 3–9 h and 1–11 h respectively). In vivo evaluation studies of metronidazole and its twin esters **3a–d** in mice and **3b** in rabbits revealed that the prodrugs have been absorbed almost unhydrolyzed with considerable higher plasma level. Antiparasitic activity of the synthesized compounds was evaluated in mice against *Giardia muris*, the prodrug **3b** showed improved antigiardial activity compared to the parent drug. These results suggest that the synthesized identical twin esters **3a–d** may be useful as a promising new prodrug form of metronidazole for oral drug delivery. © Elsevier, Paris

metronidazole / identical twin prodrugs / lipophilicity / hydrolysis kinetics / in vivo evaluation / antigiardial activity

1. Introduction

For more than a decade, increasing interest has been directed toward optimization of pharmacological and pharmacokinetic properties of existing drugs through chemical modifications. A promising approach in this respect is the development of prodrugs [1–6]. Several ester and hemiester prodrugs of metronidazole were reported in an effort to enhance its water solubility for parentral administration, chemical stability, membrane permeability and to diminish the susceptibility to enzymatic degradation [7–12]. The hemiester prodrugs showed limited stability in aqueous solutions and slow bioconversion to metronidazole [10, 11]. The major reason for the unfavorable properties of such hemiester prodrugs is partly due to the presence of terminal ionized carboxylic groups and the lack of in vitro plasma catalysis [12, 13].

Recently identical twin esters have become a common strategy for the production of prodrug forms, in which two molecules of the same drug are linked together through a spacer and after administration are metabolized into the parent drug. These modifications usually impart improvements in some deficient physicochemical properties, aiming at increasing the concentration of the drug at the site of action, prolong its duration of action or reduce its toxicity [14, 15].

In this study, a new series of identical metronidazole twin ester prodrugs **3a–e** was synthesized using different

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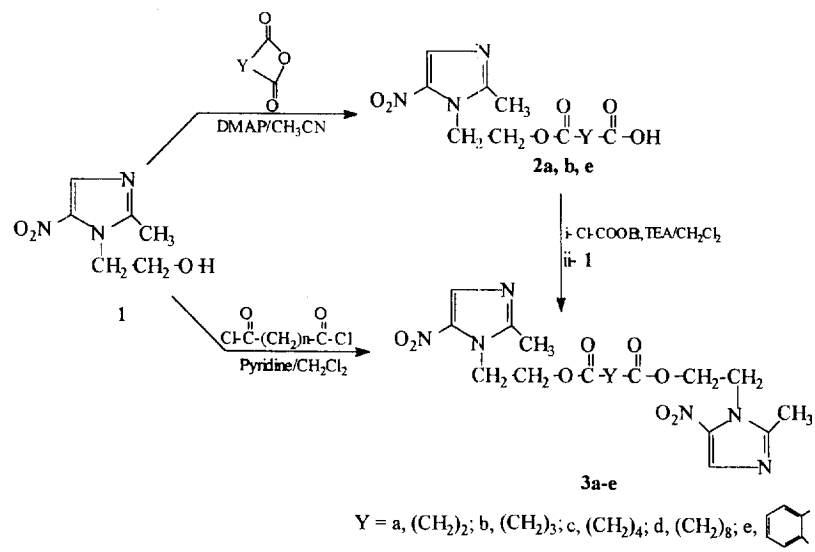

Figure 1.

dicarboxylic acids as spacer arm: succinic, glutaric, adipic, sebacic, or phthalic acid. As prodrug forms, the new derivatives should be adequately stable in aqueous solutions and represent good substrates for plasma esterases to rapidly release one molecule of metronidazole and a sustained release of the other. The variation of the acyl spacer of the selected dicarboxylic acid intented to evaluate the influence of a pro-moiety on the physicochemical properties as well as in vitro and in vivo rates of prodrug bioconversion. These systematic studies are intended to provide more information about the potential utility of identical twin ester prodrug forms for oral drug delivery. In addition, the antiparasitic activities of the developed target esters were evaluated against Giardia muris in comparison to an equivalent amount of metronidazole to investigate the possible improvement in activity with respect to the new physicochemical properties of the prepared prodrugs.

2. Chemistry

The studied metronidazole twin esters (**3a–e**) were synthesized by interaction of metronidazole (**1**) with the respective dicarboxylic acid anhydride or dichloride (*figure 1*). The synthesis of compounds **3a** and **3e** necessitate prior preparation of the precursors metronidazole hemiesters of succinic and phthalic acids (**2a,e**), by treatment of metronidazole with the corresponding freshly prepared anhydrides [16] in presence of catalytic amount of 4-dimethylaminopyridine (DMAP) [17]. Subsequent esterification of the resulting hemiesters **2a** and **2e** with metronidazole was achieved via formation of their mixed anhydrides using ethyl chloroformate and triethylamine (TEA) in methylene chloride. The moderate yields of the final products as well as the unavailability of dicarboxylic acid anhydrides limit the application of this method for preparation of the other identical derivatives. Accordingly, the twin esters **3b–d** were obtained in good yield via one step acylation reaction of metronidazole with the respective dicarboxylic acid dichloride in presence of pyridine at ambient temperature.

The purity of the synthesized compounds was assessed by TLC as well as HPLC and their structures were verified on the basis of elemental and spectroscopic methods of analysis.

The IR spectra of twin esters derived from aliphatic dicarboxylic acids **3a–d** showed carbonyl stretching bands in a range of 1735–1741 cm^{-1}, while that of phthalic acid **3e** appeared at 1719 cm^{-1}, as a result of conjugation with the aromatic moiety.

The pattern of methylenic protons of aliphatic dicarboxylic acids of compounds, **3a–d**, in ^{1}H-NMR spectra was generally affected by the length of the spacer chain. In compound **3a** they appeared relatively down field at δ = 3.33 ppm as singlet, while compounds **3b–d** showed two separate multiplet signals at $\delta \sim 1.15$–1.85 and 2.15–2.2 ppm. The phenylene protons of compound **3e** appeared at δ = 7.6 ppm as singlet signal.

3. Results and discussion

3.1. Lipophilicity

Reversed-phase thin layer chromatography (RP-TLC) is now a well-accepted method for determination of lipophilicity within a homologous series of compounds [18, 19]. The calculated chromatographic parameter, R_m value, provided a convenient measurement of log P [20]. Accordingly the lipophilicity of the synthesized twin esters **3a–e** as well as metronidazole was determined using RP-TLC method. Their R_f values were first determined using impregnated silica gel plates (1-octanol/acetone, 3% v/v) as stationary phase and mixtures of aqueous phosphate buffer (pH = 7.4) in methanol (50–90% v/v) as eluent. The obtained R_f values were used in calculation of the corresponding R_m values according to the following equation:

$$R_m = \log(1/R_f - 1) \quad (1)$$

The results are presented in *table I*. The R_m values at 0% methanol were then calculated from the linear regression equations ($r = 0.999 - 0.974$) derived by correlating the calculated R_m value of each twin ester prodrug to the respective percentage of methanol. As evident from *table I*, the target twins are quite lipophilic relative to metronidazole. This should increase the transport across biological membrane and into the cells by passive diffusion [21].

It is noteworthy that, within the identical twins, **3a–d**, derived from the aliphatic dicarboxylic acids, a positive linear dependence of ΔR_m values on the number of carbon atoms of the aliphatic chain spacer (C) as expressed by the following equation:

$$\Delta R_m = -0.847 + 0.238\,C \quad (2)$$
$$n = 4,\ r = 0.998$$

3.2. Stability in aqueous buffer solutions

The kinetics of hydrolysis of the synthesized twin ester prodrugs **3a–e** were studied in aqueous buffer solutions of pH 1.2 (non enzymatic Simulated Gastric Fluid, SGF) and isotonic phosphate buffer of pH 7.4 at 37 °C using HPLC. The different peaks in HPLC were identified by comparison of their retention times with those of authentic samples of metronidazole and its hemiesters chromatographed under the same conditions. The disappearance of the tested compounds displayed pseudo first-order kinetics over the investigated pH and temperature. As evidenced by HPLC the hydrolysis of the studied compounds was found to proceed in two step reaction (*figure 2*), whereby one of the ester moieties hydrolyzes to yield metronidazole and its hemiester. The later undergoes spontaneous hydrolysis to metronidazole (**1**) and the corresponding dicarboxylic acid. *Figure 3* shows a representative of HPLC chromatogram of **3b** and *figure 4* illustrates the time course for hydrolysis of compound **3b** at pH 1.2 and pH 7.4.

The hydrolysis rate constants k_1 and k_2 of the twin ester prodrugs **3a–e** were calculated using the MULTI computer program [22]. As a general pattern, the synthesized derivatives showed relative stability in the investigated aqueous solutions and the degradation rates at pH 7.4 are

Table I. R_m values of the synthesized twin ester prodrugs **3a–e** and metronidazole **1**.

Compound	% of methanol in phosphate buffer (v/v)						
	10	15	20	30	40	50	0
3a	–	0.038	0.023	–0.005	–0.034	–	0.081 (0.999)
3b	–	0.081	0.0313	–0.075	–0.1997	–	0.254 (0.999)
3c	0.378	–	–	0.1798	0.111	–	0.464 (0.997)
3d	–	1.126	1.04	0.807	0.565	–	1.481 (0.999)
3e	–	0.283	0.229	1.38	0.0069	–	0.450 (0.999)
1	–	–0.124	–	–	–0.227	–0.325	–0.035 (0.974)

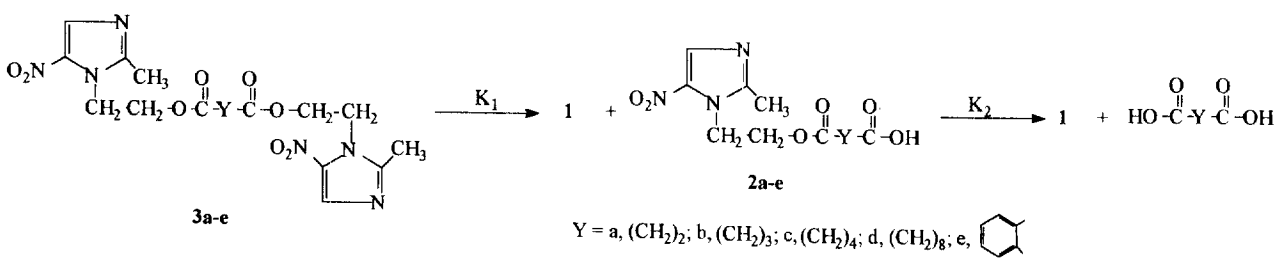

Figure 2.

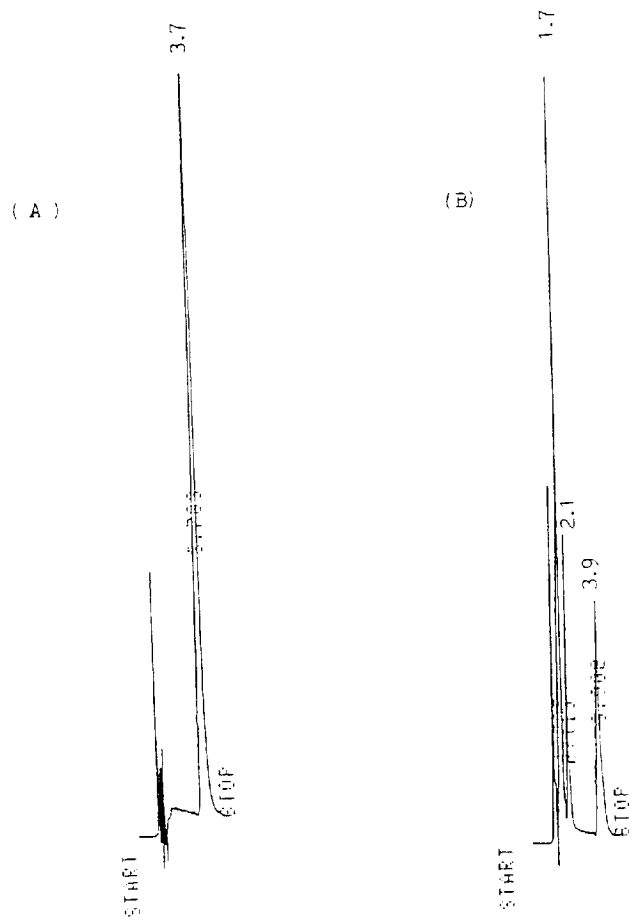

Figure 3. HPLC chromatogram of **3b** in isotonic phosphate buffer, PH 7.4, at 37 °C at time 0 min (A) and after 24 h (B). The peak with a retention time of 3.7 min represents **3b**, while those with retention times of 2.1 and 1.7 min represent **2b** and **1**, respectively.

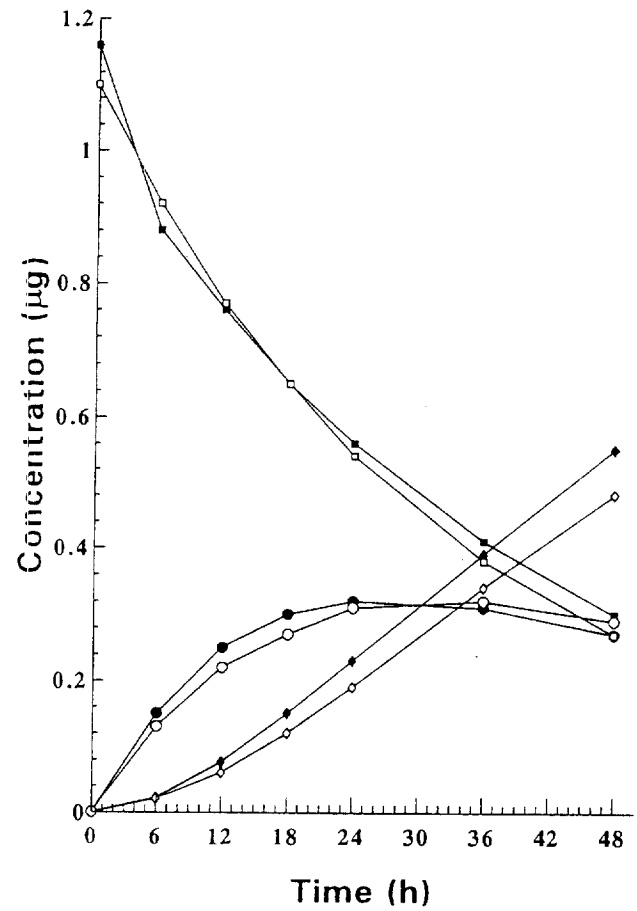

Figure 4. Time course for compound **3b** (squares), the intermediate hemiester **2b** (circles) and metronidazole (diamonds) in the degradation of **3b**, in aqueous buffer solution of pH 1.2 (open symbols) and pH 7.4 (closed symbols) at 37 °C.

slightly accelerated ($t_{1/2}$ ~ 13–40 h) than those observed in SGF of pH 1.2 ($t_{1/2}$ ~ 25–72 h). The second hydrolysis rate (k_2) is much more enhanced than the first (k_1) in both aqueous solutions (*table II*). This is consistent with the reported enhancing effects of the free carboxylic group of the dicarboxylic acids on the hydrolysis rates [13]. Both degradation rates (k_1 and k_2) of the twin ester prodrugs **3a–d** and their hemiesters **2a–d** affected by the promoiety chain length, where slow hydrolysis was observed as the number of carbon atoms in the aliphatic chain of the acyl spacer increases. No attempts were made to investigate the mechanism of degradation specially those of metronidazole hemiesters of dicarboxylic acid, however, previous investigations revealed that such types of compounds underwent hydrolysis without intramolecular reaction [12].

3.3. Stability in biological media

The hydrolysis kinetics of the prepared twin ester prodrugs **3a–e** were also studied in 80% human plasma and 20% rat liver homogenate at 37 °C. The degradation process was found to conform closely with pseudo first-order kinetics, and the parent drug was released in quantitative amounts. The degradation pattern appears to proceed as that occurred with aqueous buffer solutions, (*figure 2*), and the data are listed in *table II*. A representative example of the time course for the degradation products observed in the investigated biological media illustrated in *figure 5*. Contrary to the hydrolysis in aqueous buffer solutions, the investigated twin ester prodrugs **3a–d** undergo rapid plasma cleavage ($t_{1/2}$ ~ 10–150 min) and a slow hydrolysis rate was ob-

Table II. Rate constants (h^{-1}) for the hydrolysis of metronidazole twin ester prodrugs **3a–e** in aqueous buffer solutions, 80% human plasma and 20% rat liver homogenate at 37 °C.

Compound	pH 1.2		pH 7.4		Plasma		Liver homogenate	
	K_1 $t_{1/2}$ (h)	K_2 $t_{1/2}$ (h)	K_1 $t_{1/2}$ (h)	K_2 $t_{1/2}$ (h)	K_1 $t_{1/2}$ (h)	K_2 $t_{1/2}$ (h)	K_1 $t_{1/2}$ (h)	K_2 $t_{1/2}$ (h)
3a	0.0280 (24.75)	0.4572 (1.52)	0.0516 (13.43)	0.5655 (1.23)	0.2644 (2.62)	0.0782 (8.86)	0.7522 (0.92)	0.0887 (7.81)
3b	0.0249 (27.83)	0.0899 (7.71)	0.0414 (16.74)	0.3673 (1.89)	1.1427 (0.61)	0.0970 (7.14)	2.1947 (0.32)	0.0638 (10.86)
3c	0.0115 (60.26)	0.0454 (15.26)	0.0197 (35.18)	0.0614 (11.29)	2.4730 (0.28)	0.2382 (2.91)	4.6226 (0.15)	0.2428 (2.85)
3d	0.0096 (72.19)	0.0181 (38.29)	0.0173 (40.06)	0.0346 (20.03)	3.9760 (0.17)	0.2036 (3.40)	11.0199 (0.06)	0.9229 (0.75)
3e	0.0121 (57.27)	1.0457 (0.66)	0.0441 (15.71)	1.1146 (0.62)	0.0559 (12.39)	0.0172 (40.29)	0.0222 (31.22)	0.4370 (1.59)

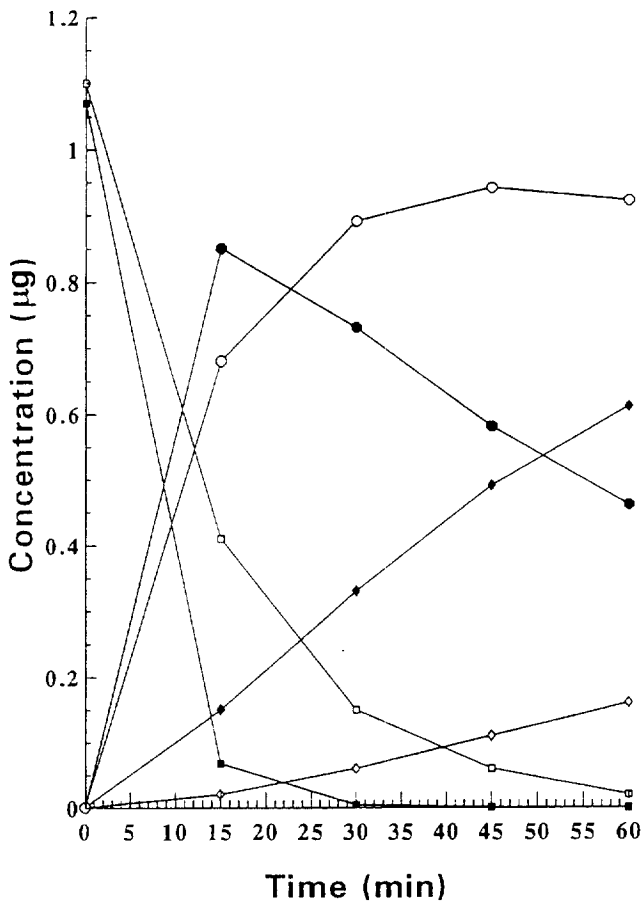

Figure 5. Time course for compound **3c** (squares), the intermediate hemiester **2c** (circles) and metronidazole (diamonds) in the degradation of **3c**, in 80% human plasma (open symbols) and 20% rat liver homogenate (closed symbols) at 37 °C.

served for their hemiesters precursors (3–9 h). Such delayed degradation rate (k_2) can be attributed to the lack of in vitro plasma catalysis as has been previously described for analogous hemiesters [12, 23]. The enhanced pronounced effect on the degradation rate (particularly k_1) of the tested compounds reflect the significant of enzymatic catalysis.

The susceptibility of the synthesized prodrugs **3a–d** to human plasma enzymes enhances as the distance between the two ester moieties increases. The variation in the rates of degradation of the resulting hemiesters **2a–d** was found to be also related to the distance between the terminal ionized carboxylic group and the ester bond to be hydrolyzed. On other words, a decrease in the length of the pro-moiety affords a reduction in cleavage rates. Consequently, the observed delayed degradation rates of the resulting hemiesters **2a–d** might serve as sustained release prodrug form of metronidazole. These results are in accord with reported observations on analogous metronidazole hemiesters [12].

The bioconversion of the studied twin ester prodrugs **3a–d** in rat liver homogenate enzyme system ensued in a similar pattern with enhanced rates relative to those in human plasma. This can be owed to the much higher esterase activity in rat liver compared to human plasma [21].

The twin ester prodrug derived from phthalic acid **3e** was studied as a representative example for aromatic dicarboxylic acid. It is apparant that, this derivative is not substrate for enzyme estreases as indicated from the observed slow release of the parent drug in the investigated enzyme systems ($t_{1/2}$ = 12.4 h and 31.2 h), relative to the corresponding aliphatic twin esters (*table II*). The

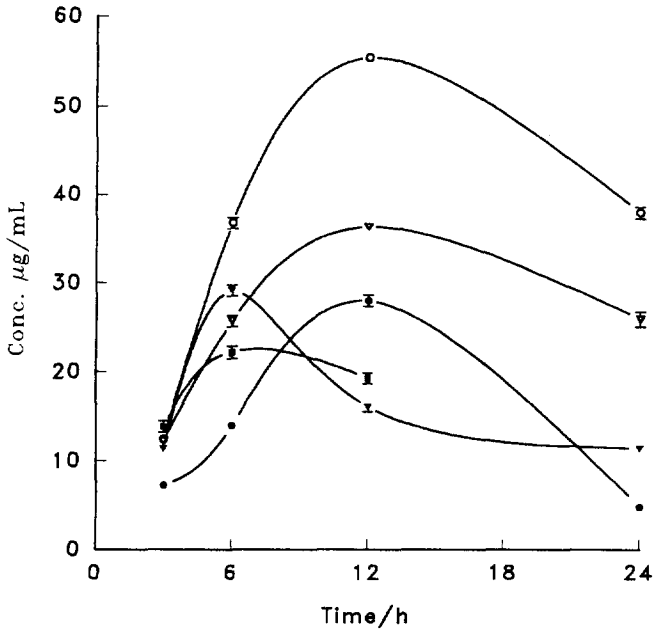

Figure 6. Plasma levels of metronidazole after oral administration of **3a** (closed circles), **3b** (open circles), **3c** (closed triangles), **3d** (open triangles) and metronidazole (closed squares) to mice (dose: 0.175 mmol/kg). Each point represents the mean ± SE (n = 3).

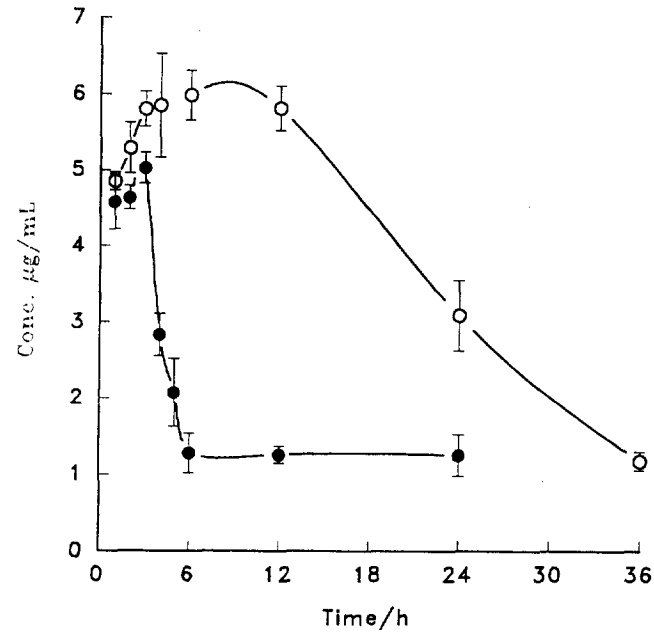

Figure 7. Plasma levels of metronidazole after oral administration of **3b** (open circles) and metronidazole (closed circles) to rabbits (dose: 0.175 mmol/kg). Each point represents the mean ± SE (n = 3).

retarded susceptibility of this compound to enzymatic hydrolysis may be attributed to some steric factors.

3.4. Bioavailability study

The synthesized twin ester prodrugs **3a–d** and the parent drug were evaluated for their in vivo bioavailability after oral administration in mice at different time intervals (*figure 6*). The prodrugs **3b–d** resulted in higher plasma levels of metronidazole up to 10 h postdosing as compared with an equivalent dose of the parent drug. In agreement with the observed in vitro results, compound **3a** attained highest plasma level of metronidazole only after 12 h. Moreover, the parent drug could not be detected after 12 h in metronidazole-treated mice, whereas blood samples of the prodrugs-treated mice show considerable concentrations of metronidazole up to 24 h indicating a sustained release of the parent drug.

The twin ester prodrug **3b** was chosen for in vivo evaluation study after oral administration in rabbits in comparison with equivalent dose of metronidazole (0.175 mmol/kg). The rabbits treated with the twin ester prodrug **3b** showed higher plasma level (*figure 7*) with sustained release of the parent drug (6 μg/mL for up to 12 h compared with 5 μg/mL for 3 h from equivalent dose of metronidazole). In both cases the results indicated that, the prodrugs were almost absorbed unhydrolyzed and the hydrolysis of the ester bonds may occur mainly in circulating system.

3.5. Antigiardial activity

The in vivo antigiardial activity of the twin prodrugs **3a–e** and metronidazole was evaluated in mice against *Giardia muris* according to the protocol described in the experimental section, to investigate the possible enhancement in activity with respect to the new physicochemical properties of the synthesized twin esters prodrugs. A single dose of 30 mg/kg was reported for metronidazole [24] and chosen as a reference control. The tested identical twin prodrugs **3a–e** were given in a dose level containing an equivalent amount of the parent drug.

The results obtained are expressed as % of cured mice and listed in *table III*. It appeared that, the twin ester prodrug derived from glutaric acid **3b** showed superior antigiardial activity than that obtained with the parent drug and the activity of the prodrug **3a** is equivalent to that of metronidazole. Meanwhile, the remaining identical twin esters **3c–e** however exhibited lower antigiardial activity compared to the parent drug.

Table III. In vivo antiparasitic activity of the synthesized twin ester prodrugs **3a–e** and metronidazole **1**, against *Giardia muris* in mice.

Compound	Dose [mg (mmole) / kg]	Equivalent metronidazole content [%w / w]	Cured mice	Uncured mice	% of cured mice
3a	36.78 (0.0875)	80.68	10	2	83.33
3b	38.01 (0.0875)	78.08	12	–	100.00
3c	39.56 (0.0875)	74.76	7	5	58.33
3d	44.50 (0.0875)	67.32	5	7	41.67
3e	41.34 (0.0875)	72.46	5	7	41.67
1	30.00 (0.175)	100.0	10	2	83.33

4. Conclusion

The in vitro evaluation of the twin ester prodrugs **3a–e** indicated that these derivatives were more lipophilic than the parent drug and stable enough in SGF as well as at physiological pH to be absorbed intact. The prodrugs were bioconverted to metronidazole, with rapid release of the first molecule and slow hydrolysis of the other. The degradation of the synthesized prodrugs seemed to be controllable by their structural parameters as evidenced by the kinetic data. Furthermore, in vivo evaluation study of the twin ester prodrugs **3a–d** and metronidazole, given orally to mice and rabbits indicated that, the prodrugs absorbed almost unhydrolyzed with considerable higher plasma level and sustained release of the parent drug. In addition, the twin prodrugs involved in the current investigation displayed matchable or improved activity against *G. muris* compared with the parent drug, metronidazole.

5. Experimental protocols

Metronidazole was obtained from Alexandria Co. for drugs and chemical industries, Alexandria, Egypt. All other chemicals were of commercial grade except the HPLC solvents and the buffer reagents (analytical grade). Melting points were determined on an electrothermal melting point apparatus (Fa. Sturat Scientific, England), and were uncorrected. Precoated silica gel plates (kiesel gel 0.25 mm, 60G F254, Merck) were used for thin layer chromatography. Developing solvent system of chloroform/acetone/triethylamine (5.5:4.5:0.5) was used and the spots were detected by UV at 254 nm. IR spectra(KBr disc) were recorded on IR-470 Shimadzu spectrometer, Japan. ^{1}H-NMR spectra were scanned on a Varian EM-360 L NMR spectrometer (60MHZ) USA, using $CDCl_3$ or DMSO-d_6 as a solvent. Chemical shifts are expressed in δ (ppm) relative to TMS as an internal standard. Elemental analyses were performed at the Department of Chemistry, Faculty of Science, Assiut University, Assiut, Egypt.

HPLC system consisting of a pump (KNAUER HPLC pump 64, Germany), a variable-wavelength detector (KNAUER), a reversed-phase HPLC column (stainless steel (25 x 0.5 cm i.d.) C-18 Eurospher 80) connected with a cartidge guard column, a Shimadzu C-R 6A chromatopac recording integrator, and a 20-μL injection loop was used. Mobile phase systems of acetonitrile, methanol and 0.1% phosphoric acid were used. The ratio of acetonitrile: 0.1% phosphoric acid was adjusted in order to give a retention time of 3.5–7 min for the synthesized derivatives and of 1.5–2.7 min for the degradation products (**1** and **2a–e**), however, methanol content was 10% in all developing systems. The column effluent was monitored at λ = 270 nm and the flow rate was 1 mL/min. Quantitation of the eluted compounds was done from peak height measurements in relation to those of standards chromatographed under the same conditions. For in vivo monitoring of metronidazole, a reversed phase HPLC column (stainless steel (25 x 0.46 cm i.d.) C-8 Hypersil BDS) was used. The mobile phase consists of acetonitrile, 0.1% phosphoric acid (100:900) containing 0.1 mL TEA to afford a retention time of 5.5 min for metronidazole.

Antigiardial activity of the synthesized compounds was performed at the Department of Parasitology, Faculty of Medicine, Assiut University, Assiut, Egypt.

5.1. General procedure for preparation of metronidazole hemiesters (2a,b,e)

Metronidazole (23.4 mmol) was dissolved in 350 mL of acetonitrile and 23.4 mmol of the respective freshly prepared acid anhydride followed by 4-dimethylamino-pyridine (2.4 mmol) were added. The mixtures were left at ambient temperature for 48 h or until the reaction was completed as evidenced by TLC. The products were precipitated either during the reaction (**2e**) or after solvent evaporation and addition of cold water (**2a** and **2b**).

1-[2-(2-Methyl-5-nitroimidazol-1-yl)ethyl] butandioate (2a): Yield 75%, m.p. 105–106 °C (aq. ethanol), reported 106–107 °C [11], ^{1}H-NMR ($CDCl_3$): 2.55 (7H, s, CH_3 and $COCH_2CH_2CO$); 4.5 (4H, m, OCH_2CH_2N) and 7.9 (1H, s, imidazole-H).

1-[2-(2-methyl-5-nitroimidazolyl-1-yl[ethyl] pentandioate (2b): Yield 53%, m.p. 103–104 °C (aq. ethanol) reported 104–105 °C [11], ^{1}H-NMR ($CDCl_3$): 1.95 (2H, m, $CH_2CH_2CH_2$); 2.3 (4H, t, $CH_2CH_2CH_2$); 2.6 (3H, s, CH_3); 4.5 (4H, m, NCH_2CH_2O), 8.0 (1H, s, imidazole-H) and 9.0 (1H, bs, COOH).

2-[(2-Methyl-5-nitroimidazol-1-yl)ethyl] phthalate (2e): Yield 90%, m.p. 171–173 °C (aq. ethanol); IR (cm^{-1}): 3530 br (OH);

1728 (C=O ester); 1696 (C=O acid) 1487, 1385 (NO_2) and 1255, 1133 (C–O–C). ^{1}H-NMR (DMSO-d_6): 2.3 (3H, s, CH_3); 4.5 (4H, brs, OCH_2CH_2N); 7.5 (4H, m, C_6H_4) and 7.9 (1H, s, imidazole-H). Found: C, 52.79; H, 4.10; N, 13.15. Calculated for $C_{14}H_{13}N_3O_6$: C, 52.67; H, 4.10; N, 13.16.

*5.2. General procedure for synthesis of metronidazole twin esters (**3a,e**)*

Metronidazole hemiester **2a** or **2e** (4.0 mmol) were suspended in 50 mL dried methylene chloride, triethylamine (4.0 mmol, 0.8 mL) was added and the stirred mixture was cooled (0–5 °C). Ethylchloroformate (4.0 mmol, 0.44 mL) was added dropwise and stirring was continued for 30 min. A suspension of metronidazole (4.0 mmol) in dry methylene chloride (5.0 mL) was then added and the resulting clear solution was stirred at room temperature for further 5 h or until the reaction was completed (TLC). The solvent was evaporated under reduced pressure and the product was recrystallized from methanol.

*1,4-Bis[2-(2-methyl-5-nitroimidazol-1-yl)ethyl] butandioate (**3a**):* Yield 58%, m.p. 128–130 °C, IR (cm^{-1}): 1735 (C=O); 1465, 1364 (NO_2) and 1259, 1148 (C–O–C). ^{1}H-NMR (DMSO-d_6): 2.5 (6H, s, $2CH_3$); 3.3 (4H, s, $COCH_2CH_2CO$); 4.35 (8H, m, $2OCH_2CH_2N$) and 7.9 (2H, s, 2-imidazole-H). Found: C, 45.17; H, 4.93; N, 19.39. Calculated for $C_{16}H_{20}N_6O_8$: C, 45.29; H, 4.75; N, 19.81.

*Bis[2-(2-methyl-5-nitroimidazol-1-yl)ethyl] phthalate (**3e**):* Yield 53%; m.p. 133–135 °C; IR (cm^{-1}): 1719 (C=O): 1472, 1379 (NO_2) and 1256, 1139 (C–O–C). ^{1}H-NMR ($CDCl_3$): 2.5 (6H, s, $2CH_3$); 4.65 (8H, s, $2OCH_2CH_2N$); 7.65 (4H, s, C_6H_4) and 7.95 (2H, s, 2 imidazole-H). Found: C, 51.35; H, 4.34; N, 17.47. Calculated for $C_{20}H_{20}N_6O_8$: C, 50.85; H, 4.27; N, 17.79.

*5.3. Synthesis of metronidazole twin esters (**3b–d**)*

A solution of the respective dicarboxylic acid dichloride (5 mmol) in methylene chloride (15 mL) was added dropwise to a suspension of metronidazole (1.71 g; 10 mmol) and pyridine (0.5 mL) in 20 mL methylene chloride and the resulting clear solution was stirred overnight at room temperature. The solvent was concentrated under reduced pressure and the products were filtered, washed well with ethanol and recrystallized from methanol.

*1,5-Bis[2-(2-methyl-5-nitroimidazol-1-yl)ethyl] pentandioate (**3b**):* Yield 74%; m.p. 114–115 °C; IR (cm^{-1}): 1741 (C=O); 1476, 1352 (NO_2) and 1254, 1185 (C–O–C); ^{1}H-NMR ($CDCl_3$): 1.85 (2H, m, $CH_2CH_2CH_2$); 2.2 (4H, t, J = 6 Hz, $CH_2CH_2CH_2$); 2.5 (8H, m, $2OCH_2CH_2N$) and 7.9 (2H, s, 2 imidazole-H). Found: C, 46.76; H, 5.18; N, 19.40. Calculated for $C_{17}H_{22}N_6O_8$: C, 46.58; H, 5.06; N, 19.17.

*1,6-Bis[2-(2-methyl-5-nitroimidazol-1-yl)ethyl] hexandioate (**3c**):* Yield 81%; m.p. 116–117 °C; IR (cm^{-1}): 1735 (C=O); 1452,1381 (NO_2); and 1258, 1153 (C–O–C); ^{1}H-NMR ($CDCl_3$): 1.5 (4H, m, $COCH_2(CH_2)_2CH_2CO$); 2.15 (4H, m, $COCH_2(CH_2)_2CH_2CO$; 2.85 (6H, s, $2CH_3$); 4.5 (8H, m, $2OCH_2CH_2N$) and 7.92 (2H, s, 2 imidazole-H). Found: C, 47.70; H. 5.50; N, 18.15. Calculated for $C_{18}H_{24}N_6O_8$: C, 47.79; H, 5.35; N. 18.58.

*1,10-Bis[2-(2-methyl-5-nitroimidazol-1-yl)ethyl] decandioate (**3d**):* Yield 79%; m.p. 84–85 °C; IR (cm^{-1}): 1736 (C=O); 1476, 1380 (NO_2) and 1258, 1149 (C–O–C). ^{1}H-NMR ($CDCl_3$): 1.3 (12H, m, $COCH_2(CH_2)_6CH_2$); 2.15 (4H, t, J = 7 Hz, $COCH_2(CH_2)_6CH_2CO$); 2.55 (6H, s, $2CH_3$); 4.5 (8H, m, $2OCH_2CH_2N$) and 7.9 (2H, s, 2 imidazole-H). Found: C, 51.72; H, 6.50; N, 16.91. Calculated for $C_{22}H_{32}N_6O_8$: C, 51.96; H, 6.34; N, 16.53.

5.4. Determination of R_m values

Silica gel G60 F254 plates (10 × 15 cm) were impregnated for 5 h in 3% v/v octanol in acetone and air-dried overnight. Solutions of the tested compounds (**3a–e**) or metronidazole in acetone (1 mg/mL) were spotted at 1.5 cm intervals and the plates were then developed, in jars previously saturated with the solvent system for one hour, using aqueous phosphate buffer (pH 7.4) in methanol (50–90%) to a solvent front of 12 cm height. The developed plates were air-dried and the spots were visualized in UV-light at 254 nm. Triplicate experiments were carried out to ensure reproducibility of the results. The R_f values were determined and its mean values were used in calculation of the corresponding R_m values.

5.5. Kinetic measurements

5.5.1. In aqueous buffer solutions

Degradation rates of the synthesized derivatives (**3a–e**) in aqueous solutions, (containing 0.02% w/v Tween 80) of pH 1.2 (SGF) and isotonic phosphate buffer of pH 7.4, were determined at 37 °C. The ionic strength of the buffer solutions was adjusted to 0.5 by addition of a calculated amount of potassium chloride. The reactions were initiated by adding 250 µL of a methanolic solution (2×10^{-3} M) of the twin ester prodrugs to 2.5 mL of preheated buffer solutions in screw-capped test tubes and at appropriate intervals aliquots of 20 µL were withdrawn and analyzed by HPLC for the residual prodrugs.

5.5.2. In human plasma

Degradation studies in 80% human plasma containing isotonic phosphate buffer of pH 7.4 at 37 °C was done by adding appropriate amount of the methanolic solution of the respective prodrug to the plasma solution. The initial concentration of the esters was 2×10^{-4} M. At appropriate time intervals samples of 50 µL were withdrawn, deproteinized by mixing with 50 µL acetonitrile and centrifuged at 10^4 rpm for 5 min. Aliquots of 20 µL of the clear supernatant were analyzed by HPLC as described above.

5.5.3. In rat liver homogenates

Male wister rats livers were homogenized with ice-cooled saline to give a concentration of 40% w/v, and were then centrifuged at 15000 rpm for 15 min. The supernatant was collected in 2 mL tubes and stored at –40 °C until use. Homogenate was thawed 10 min before the experiments and diluted with saline to a concentration of 20% w/v. The hydrolysis studies in rat liver homogenate were performed as described above for 80% human plasma solution. Results of kinetic measurements are given in *table II*.

5.6. In vivo evaluation study

5.6.1. In mice

Four groups each of 18 mice (20–30 g weight) were used. Each group was sequentially classified into 6 subgroups each of 3 mice for investigation of the prodrugs **3a–d**; metronidazole and a control group. An oral dose of 0.175 mmole/kg of metronidazole or its equivalent amount of the prodrugs **3a–d** was given as suspension in 0.1% carboxy methylcellulose (CMC). The control group received only the vehicle. Blood samples of 1 mL were collected by puncture of the eye cornea using capillary tube at 3; 6; 12 and 24 h from mice of the first; second; third and fourth group respectively. The samples were directly centrifuged to separate plasma. 50 µL aliquots were then deproteininzed by addition of an equivalent amount of acetonitrile and centrifuged at 10^4 rpm for 10 min. 20 µL were then analyzed for metronidazole contents using HPLC.

5.6.2. In rabbits

Two groups each of three rabbits (1.5 kg weight) were used. Each rabbit of the first group administered orally a dose of 0.175 mmol/kg of the twin ester prodrug **3b** as suspension in 0.1% of CMC. Equivalent dose of metronidazole in 0.1% CMC was administered to the rabbits of the second group. Blood samples, each of 0.5 mL, were withdrawn from the marginal ear vein at 1, 2, 3, 4, 6, 12, 24 and 36 h centrifuged to separate plasma. 50 µL of the separated plasma were treated as mentioned above for 80% human plasma and assayed for metronidazole contents using HPLC.

5.7. In vivo antigiardial activity

Groups of 12 albino mice of 3–4 weeks age (20–30 g weight), previously checked for absence of giardia infections, were used. Each mouse was infected orally by approximately 2×10^5 cysts of freshly isolated Giardia muris suspended in 1 mL of saline according to Amin method [25]. Infection of mice, was then proved as described by Vinayak [26] and the intensity of infection was determined as described by Underdown [27]. Suspensions of the tested compounds in 0.5% CMC were given orally in appropriate dose (30 mg of metronidazole or its equivalent amount of the prodrugs, *table III*) to the divided groups of mice and the control group received only the vehicle. A standard diet was given to the animals throughout the experiment. Stools of the mice in each group were microscopically examined for infection up to 7 days. Negative results (cured animals) were reexamined by zinc sulfate floatation concentration technique [28]. Antigiardial activity of the tested compounds was calculated as the percentage of cured mice in each group.

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