Amoebicidal *in Vitro* Activity Shown by Some Metronidazole Analogues: Biological Response-Reduction Potential Correlation

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INTRODUCTION

Nitroimidazoles are amongst the most studied compounds as antimicrobials (1). Metronidazole (1) a nitroimidazole is no doubt the preferred drug in the treatment of Amoebiasis an endemic disease caused by *E. histolytica* (2,3). Most of the literature reports related to the mode of action of nitroimidazoles are on Trichomonas and Clostridium, where it has been shown that nitroimidazoles act as electron acceptors during the metabolic cycle of microorganisms (4,5). Pharmacological activity of nitroimidazoles might then be explained in terms of the reduction potential and also on the compound lipophilic character. Therefore it is important to evaluate biological response associated to new compounds in terms of parameters that may be responsible for the activity (6,7). In this study, results of pharmacological *in vitro* tests on *E. histolytica* of a series of imidazolyl aryl ketones **3a-e** and metronidazole analogues **4a-e** (Fig. 1) are presented.

MATERIALS AND METHODS

Imidazolyl arylketones **3a-e** and metronidazole analogues **4a-e** used in this investigation were prepared from 2-methyl-5-nitro imidazole **2** (8), Figure 1. The culture of *Entamoeba histolytica* was pathogenic stock H1-IMSS.

Preparation of *E. histolytica* Culture

E. histolytica was grown under anoxic conditions following Diamond procedure (9,10). Trophozoites were cultured during their log growing phase, (48 h) after being seeded. Trophozoites (approximately 30,000) were washed with TYI-S-33 medium, placed in a well plate and incubated for 2 h at 37°C.

Assay Dose-Response

Drug samples were prepared by dissolving 2 mg of each compound in 100 μl DMSO and water added to reach a required concentration (0.5, 1.0, 2.0, 5,0, 10, 16 and 30 μg/ml). Pure DMSO and metronidazole solutions (similar concentrations as indicated before) were prepared as blanks. After innoculation with a preselected compound, amoebae were again incubated at 37°C for 4, 8 and 12 h. After removal of remaining drug-containing broth, culture was thoroughly fixed to the well plate surface with a 3.7% formaldehyde solution during 1 h at 37°C. Amoebae were then stained with 100 μL 0.1% methylene blue and borate (0.5 M) solution for 10 min. Fixed dye was extracted from cells by adding 100 μl of a 0.1 N HCl aqueous solution at ambient temperature, allowing contact for 20 min. Methylene blue solutions obtained were then read on a UV spectrophotometer. A calibrating curve (prepared by reading at λ_{max} = 630 dye solutions) showed dye concentration to be proportional to the number of viable amoebae which remained fixed in each well.

Each treatment was randomly assigned to microorganisms cultures and an average of 6 determinations were made for each experiment. Standard deviation σ was calculated for the population and found to be 0.060891 (σ from all spectrophotometric assays were considered).

Electrochemical Determinations

Experiments were performed in a five entries electrochemical cell designed to keep a constant nitrogen atmosphere. The working electrode was a platinum 0.283 cm^2 area disk, a platinum gauze served as counter electrode. An Ag/Ag^+ electrode was used as reference. All potentials are reported with reference to the (Fc/Fc^+) ferrocene/ferrocenium pair in agreement with the IUPAC (11). Solvent was acetonitrile containing 0.1 M tetraethylammonium fluoroborate as supporting electrolyte. In all cases a 0.002 M concentration of electroactive species was used (12).

RESULTS AND DISCUSSION

Pharmacological Tests

Regression analysis of the calibration curve gave equation 1, which determined the number of viable amoebae left after each treatment (blanks included).

$$(\text{No. of live amoebae})(1.87 \times 10^{-3}) - (0.2893) = \text{Abs}\,\lambda_{630} \quad (1)$$

The difference between number of viable amoebae in the blank (DMSO) and those in each experiment gave the number of dead amoebae. Relative activity of each analogue in turn was calculated from the ratio of dead amoebae obtained during each compound treatment and dead amoebe by metronidazole. Biological response values reported on this study refer to the 4 h drug treatment, since no significative change occured after this time. Three different modes of biological behaviour were exhibited by tested nitroimidazole derivatives.

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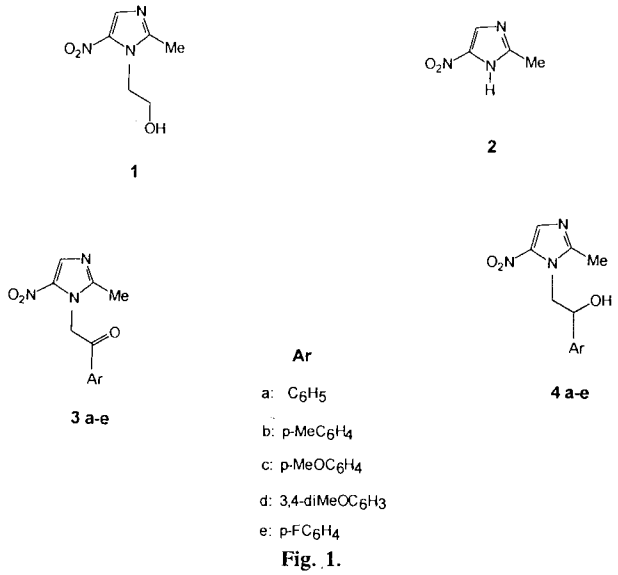

Fig. 1.

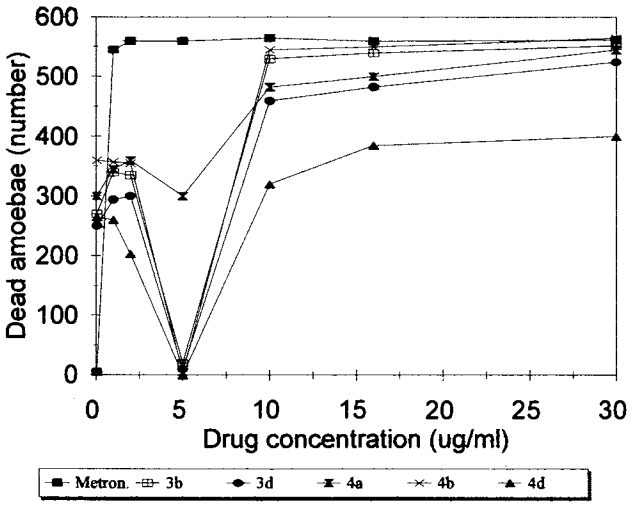

Figure 3. Amoebicidal behaviour shown by compound 3b, 3d, 4a, 4b, 4d and metronidazole.

1. Compounds **3a**, **3c**, **3e** as well as the alcohol **4e** gave a behaviour pattern similar to that of metronidazole. Compound **3c** showed an almost nil activity at a minimum concentration followed by a sudden activity increase until the maximum amoebicidal effect (MAE) was reached. In turn, compounds **3a**, **3e** and **4e** showed a gradual increase in activity until the MAE was obtained. From this maximum point on, biological activity remained constant and was independent of compound concentration as shown in Figure 2.
2. Hydroxy derivative **4c**, showed a maximum activity at a concentration of 2 μg/ml equivalent to 75% that shown by metronidazole on same conditions, Figure 2. The salient feature of this compound is that an increase in the administered dose caused an activity decrease contrary to an expected activity increase in accordance to the proposed mechanism of action, i.e. reduction of the nitro moiety (13).
3. Ketones **3b** and **3d** as well as the hydroxy derivatives **4a**, **4b** and **4d** reached a first maximum antimicrobial activity at a relatively low concentration as compared to other antimicrobials. Thus, at 0.5–2 μg/ml, they showed roughly half metronidazole activity (Fig. 3). As concentration raised, activity slightly diminished in compounds **3d** and **4a**, becoming nil in **3b**, **4b** and **4d**. An interesting fact is that the inflection point was approximately 5 μg/ml, i.e., very close to the MAE reported for metronidazole. After this point biological activity increased as a function of drug concentration.

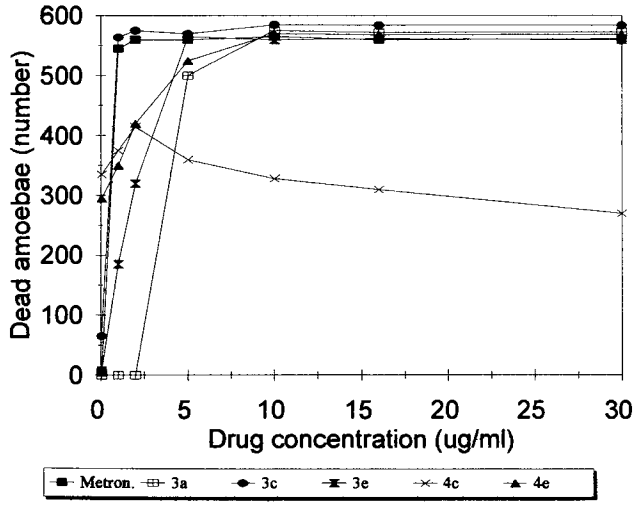

Figure 2. Amoebicidal behaviour shown by compound 3a, 3c, 3e, 4c, 4e and metronidazole.

The observed experimental fact with compound **4c** suggests that in this case drug-receptor interaction has been somehow blocked probably due to saturation at the receptor site. Alternatively, transformation of compound **4c** into in-

Table I. Activity, Potency and $E_{pc/2}$ Values for Nitroimidazoles Structurally Related to Metronidazole

Compound	% Relative* Activity[a]	Potency[b]	Molar Potency[c]	$-E_{pc/2}$ (20 mV/s)
3 a	0.98	0.2041	2.59	1651.01
3 b	0.78	0.9030	3.167	1495.00
3 c	1.02	0.9822	4.422	1523.64
3 d	0.4	−0.0017	2.463	1743.43
3 e	1.0	0.3010	2.721	–
4 a	0.52	0.6810	3.074	–
4 b	0.95	0.7780	3.195	1734.02
4 c	0.65	0.9030	3.346	–
4 d	0.0	−0.2430	2.244	1826.8
4 e	0.94	0.8360	3.22	1726.37
1	1.0	0.3187	2.552	15566.4

1 = Metronidazole.

* Relative Activity was measured at compound concentration of 5 μg/ml.

[a] Number of dead amoebae by a given compound/number of dead amoebae by metronidazole ×100.

[b] Log ($1/AC_{50}M$); AC_{50} = Compound concentration to produce *a.* 50% Amoebicidal effect.

[c] Log $(AC_{50}M/MW)^{-1}$.

Table II. Population Standard Deviation (σ_{n-1}) of Viable Amoebae Counting (Absorbance Reading at λ = 630 nm)

Conc. (μg/ml)	1	3a	3b	3c	3d	3e	4a	4b	4c	4d	4e
30	0.0774	0.0247	0.2106	0.0514	0.0305	0.0265	0.1353	0.0330	0.045	0.0682	0.0525
16	0.1748	0.0912	0.0348	0.1748	0.0288	0.1074	0.0439	0.0508	0.0423	0.0955	0.0143
10	0.0640	0.0599	0.0441	0.1515	0.0188	0.2046	0.1598	0.1430	0.2628	0.0162	0.0168
5	0.0598	0.0602	0.0289	0.0252	0.0582	0.0309	0.0280	0.0167	0.0318	0.0182	0.0148
2	0.0733	0.0541	0.0232	0.1866	0.0090	0.0665	0.0211	0.0101	0.0217	0.0180	0.0300
1	0.0531	0.0153	0.0201	0.0234	0.0112	0.0176	0.0180	0.0347	0.0064	0.0193	0.0359
0.5	0.0628	0.0287	0.0141	0.1955	0.0256	0.0648	0.0260	0.0456	0.0210	0.0436	0.0248

active metabolites by a microorganism defense mechanism cannot be ruled out. This latter explanation may be applied to compounds of point 3. In addition it might be considered that intermediate metabolites may or may no be active or that they triggered only a minor antimicrobial response and thus the observed decrease in activity.

Dose-response plots (Figs. 2 and 3) allowed for the calculation of the concentration for each compound to produce a 50% amoebicidal effect ($AC_{50}M$) compared to metronidazole. Values for potency and molar potency show that biological activity varies in the order given below. Values are given in table 1 and were obtained from expressions 2 and 3.

$$3c > 4ç, 4e > 4b > 3b > 4a > 3e > 3a > 1^* > 3d > 4d$$

*Metronidazole.

$$\text{Potency} = \text{Log}\,(1/AC_{50}M) \quad (2)$$

$$\text{Molar potency} = \text{Log}\,(AC_{50}M/MW)^{-1} \quad (3)$$

Reduction Potential Determination

The reduction potential peak [E_{pc}] and half peak reduction potential [$E_{pc/2}$] were determined for each compound through voltammograms at different potential sweep rates [10 mV/s < v < 1000 mV/s]. It was generally observed that E_{pc} and $E_{pc/2}$ are v independent at v < 50 mV/s. Half peak reduction potentials are reported here at scan rate of 20 mV/s and given in Table 1. Values for compounds **3e, 4a** and **4c** are not available due to dissolution problems.

An average reduction potential near that of metronidazole was observed on the ketone derivatives **3a-d**, exception being compound **3c**. Hydroxy derivatives **4a-d** showed reduction potential values higher than that of metronidazole. However highest activity matched minimum reduction potential value, i.e., near that of metronidazole. It thus seems possible to associate a maximum activity to a reduction potential close to the one given by metronidazole, whilst a distant values from that results in a decreased activity. A good correlation between reduction potential and biological activity was obtained on contrasting $E_{pc/2}$ values vs. antimicrobial potency for both, **3a-e** and **4a-e**, giving a correlation factor (r^2) of 0.999 and 0.972 respectively following equations 4 and 5.

$$\text{Log}(AC_{50}) = -19.589\,E_{pc/2} + 1.08 \times 10^{-2} \quad (4)$$
$$r^2 = 0.9997$$

$$\text{Log}(AC_{50}) = -8.10\,E_{pc/2} + 4.75 \times 10^{-3} \quad (5)$$
$$r^2 = 0.985$$

Very interesting was to observe an oxidation peak in the metronidazole voltammogram (E_{pa}) which suggests the possibility of reoxidising the reduced species. Similar behaviour was observed by alcohol **4d** and ketone **3d**. All other compounds showed the reduction peak only (12).

In summary, metronidazole analogues here analysed had the structural characteristics to be recognized by the metabolic cycle of the microorganism *Entamoeba histolytica* thus generating a biological response. Moreover some of the compounds exhibited amoebicidal *in vitro* behaviour comparable or slightly higher than metronidazole although it was rather surprising to find out that imadazolyl ketones displayed biological action as well.

Antimicrobial activity observed may be argued as a result of lipophilic character given by the aromatic sustituents coupled to an adequate reduction potential. The nitro functionality then played an important role in the observed antimicrobial action confirming a plausible redox mechanism of action (14).

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