RELATIVE EFFECTS OF ANTIMALARIAL DRUGS ON THE BINDING OF 17\(\beta\)-OESTRADIOL TO RECEPTORS IN RABBIT UTERUS

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Summary

The binding of 17\(\beta\) oestradiol to rabbit uterine cytosol receptors in the presence of the antimalarial drugs chloroquine, primaquine, paludrine and quinine was investigated.

All four antimalarial drugs enhanced the binding of the steroid to the receptors. At the highest concentration of antimalarial drugs that was tested, the increase in the binding of the steroid was in the order Quinine \(
\geq\)
 Primaquine \(
\geq\)
 Paludrine \(
\geq\)
 Chloroquine.

Chloroquine, however, had a dual effect. The increase in binding rose sharply from zero chloroquine concentration to a maximum effect at 0.6mM, then started to decrease until it levelled off at 3.0mM.

The significance of these results is discussed.

Key Words: Antimalaria drugs, oestradiol receptor binding

Introduction

Previous undocumented reports suggested that antimalarial drugs could interfere with normal reproductive processes; for instance the use of large doses of antimalarial drugs to terminate pregnancies.

This prompted some investigation into the possible effect of chloroquine, a common antimalarial drug, on reproductive processes. Engman and Vartanian\(^1\) reported that large doses of chloroquine caused abortion in rats. Asamoah\(^2\) found that chloroquine, 17\(\beta\) oestradiol \([1,3,5\,(10)\text{-estratriene-3, 17}\beta\text{ diol}]\) and mestranol (17\(\alpha\)-ethinyl-3-methoxy-estr-1, 3, 5 (10)-triene-17\(\beta\)-ol) each increased the activity of rat uterine peroxidase and the effect of both chloroquine and either estrogen administered together was additive. Earlier on, Lyttle and DeSombre\(^3\) had reported that estrogen administration to rats increased peroxidase activity in the uterus and the activity could be used as a marker for estrogen action. Chloroquine also enhanced the binding of 17\(\beta\) oestradiol to rabbit uterine cytosol receptors whereas it decreased that of progesterone\(^4\). If this happened in vivo, just as its effect on uterine peroxidase activity, it could offer an explanation as to the way chloroquine may cause the abortion. Chlorpromazine, a drug widely used for the treatment of schizophrenia and which has certain chemical similarities to the quinoline-type antimalarials was found to increase the binding of 17\(\beta\) oestradiol by human uterine cytosol.

At the moment, antimalarial drugs are prescribed to women living in malaria-endemic countries where the use of oral contraceptives to control birth is being encouraged. It would be desirable, therefore, to know if there is any adverse effect on such women due to a possible

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interaction of the two drugs administered concurrently.

The present endeavour is a continuation of work on an earlier report indicating that chloroquine increased the binding of 17β oestradiol to rabbit uterine cytosol; specifically the aim is to find out if other antimalarial drugs behave similarly to chloroquine.

Materials and Methods

Materials:

[2, 4, 6, 7 - H (N) ] 17β oestradiol, specific activity 100 Ci/mmol, was obtained from New England Nuclear, Boston, Mass. Tritiated chloroquine diphosphate, specific activity 164 Ci/mmol was prepared by custom tritiation of unlabelled chloroquine diphosphate, at the Radiochemical Centre, Amersham, U.K. Chloroquine diphosphate and quinine hydrochloride were obtained from Sigma Chemical Company, Mo, U.S.A. Primaquine phosphate and paludrine were obtained from Imperial Chemical Industries, England. The rest of the chemicals used were obtained from Flucka Ag, Buchs SG, Switzerland; Fisons Scientific Apparatus, England; British Drug House (AnalaR) Laboratory Reagent, England; and Sigma Chemical Co, Mo, U.S.A. Nitrogen gas was supplied by L'air Liquide, Tema, Ghana. Rabbits were obtained from Commercial Rabbitary, Tema, Ghana.

Preparation of rabbit uterine cytosol

Female rabbits aged 10–12 weeks were sacrificed and the uterus washed quickly in ice-cold 0.05M Tris-HCl buffer, pH 7.4, containing 0.05M sucrose, 0.01M mercaptoethanol, 0.01M sodium azide, 2M urea and 10% glycerol. The uteri were homogenized at 0–4 °C in 10ml of the same buffer using a teflon tissue homogenizer. The homogenized tissue was then centrifuged at 0–4 °C for 1 hour at 105 000g and the supernatant retained as the uterine cytosol. It was diluted to 0.1mg protein per ml with the homogenizing buffer. To remove endogenous steroids, the cytosol was treated with dextran–charcoal: an equivalent volume of buffered dextran–charcoal (11g Norit A and 1.1g dextran in 1 litre 0.05M Tris-HCl, pH 8.5) was centrifuged to produce a pellet, and the supernatant was discarded. Cytosol was added to this pellet, which was reasuspended by agitation. Shaking was continued at 0–4 °C for 10 min, followed by centrifugation at 1000g for 20 min. The supernatant cytosol, which was then free of endogenous steroids, was kept on ice prior to use in the binding assays.

Binding Assays

50 microliters of an ethanol solution of tritiated 17β oestradiol (10.0 Ci/ml) was dispensed into conical glass centrifuge tubes and various known amounts of 50 micromolar stock solutions of antimalarial drugs were added. The mixtures were dried over nitrogen gas to prevent oxidation of the oestradiol. 1.0ml aliquots of the steroid free uterine cytosol were dispensed into the centrifuge tubes and the contents of the tubes thoroughly mixed using a Vortex mixer. The tubes were then incubated in an ice shaker–bath (0–4 °C) for 3 hours. The unbound tritiated 17β oestradiol was removed with a pellet from 1.0ml charcoal–dextran suspension as described for the removal of endogenous steroids. 0.2ml aliquots of supernatants were then placed in glass scintillation counting vials containing 5.0ml of scintillation cocktail (100g naphthalene and 5.0g 2, 5-diphenyl oxazole dissolved in a liter of 1, 4-dioxane), left overnight in the dark to equilibrate and counted in a Parkard-Tri-Carb Liquid Scintillation Spectrophotometer, Model 3320. The experiments were run in duplicates and repeated five times for each antimalarial used. The same procedure was followed for the binding assay of chloroquine. Varied amounts of 60.98 micromolar tritiated chloroquine diphosphate (10 nCi/ml) were used.

Results

Increase in Binding of Oestradiol due to Antimalarials

Fig. 1 shows the results of incubating rabbit uterine cytosol with 5mM tritiated 17β oestradiol and various concentrations of antimalarial drugs. Because the
binding capacity of uterine cytosol preparations varied from experiment to experiment due to variations in binding capacity of the different uteri pooled for each cytosol preparation, the highest counts per min per mg protein in each experiment was assigned the value 1.0 and other counts per min per mg protein normalized accordingly in order to obtain meaningful averages of the five experiments done with different cytosol preparations.

All antimalarials increased the binding of the oestradiol to its receptors. The increase was sharp in the lower concentration ranges of antimalarials used but more gradual at higher concentrations. Chloroquine showed a dual effect; it increased the binding of oestradiol sharply from low concentrations to a maximum at 0.6mM and then started to decrease the binding until it levelled off around 3.0mM. Other antimalarials continued to cause an increase up to the highest concentrations tested. At these concentrations, quinine caused the greatest increase in the binding of oestradiol followed by primaquine, then paludrine and chloroquine in that order.

The Binding of Tritiated Chloroquine to Rabbit Uterine Cytosol

Fig. 2 shows the binding of tritiated chloroquine to uterine cytosol in the presence and absence of tritiated oestradiol, unlabelled oestradiol and unlabelled chloroquine. The results indicated that chloroquine bound to uterine cytosol just as 17β oestradiol did. Whereas the presence of unlabelled chloroquine lowered the binding of tritiated chloroquine, the presence of unlabelled oestradiol did not affect the binding capacity of the tritiated chloroquine. The total counts were, however, higher when the
cytosol was incubated with both tritiated chloroquine and tritiated estradiol.

Fig. 3 shows the Scatchard analysis of the binding of tritiated chloroquine to rabbit uterine cytosol. The dissociation constant \( k_d \) was 1.70 nM and the concentration of binding sites was \( 15 \times 10^3 \) f mol /mg protein. In the presence of 5 nM unlabelled oestradiol, the corresponding figures were 1.97 nM and \( 15.9 \times 10^3 \) f mol /mg protein respectively.

Discussion

As mentioned earlier, oral contraceptive steroids are being used by women who may of necessity take antimalarial drugs at the same time.

It is generally accepted that any departure from the recommended pattern of administration of oral contraceptives can render them less effective. Thus the simultaneous administration of oral contraceptive hormones and drugs that interact with them could render them less effective. Antibiotics such as ampicillin and rifampin and the antidepressant drug phenyl butazone have been reported to decrease the effectiveness of oral contraceptive preparations by increasing the breakdown of the estrogen component, thereby raising the frequency of unwanted pregnancies.

In this investigation, the antimalarials all increased the binding of 17\( \beta \) oestradiol to its receptors in rabbit uterine cytosol. This increased binding is likely to be "specific" binding because in the case of chloroquine which had been reported earlier, the binding constant was not affected by the presence of the antimalarial. It is conceivable that the same phenomenon could occur in vivo just as the in vivo effect of chloroquine on uterine peroxidase activity. This can upset the balance of the effect of the estrogen component, since the antimalarials have slow metabolic turnover rates, slow rate of excretion and persist for a long time in the body even after stop-

ping their therapy. Any possible effect of antimalarials on the efficacy of the oral contraceptives is at best speculative. However, if the increased binding of oestrogen to the uterus due to antimalarials should lead to an increased effect of the oestrogen component of the oral contraceptive, then even if ovulation should occur, pregnancy may not be sustained since the oestrogen component in the system would outweigh the progestin component. In this case, the antimalarials would promote the course of oral contraceptives. But over a long period of time, there could also be other adverse side effects on the general health of the subject due to the abnormal increase in the effect of oestrogens.

Chloroquine did bind to uterine cytosol but because chloroquine is an active molecule which can bind to a large number of proteins, its binding to uterine cytosol does not necessarily imply that it has specific receptors in the uterus. However, because unlabelled 17\( \beta \) oestradiol did not lower the level of binding of the tritiated chloroquine, chloroquine is unlikely to be binding to the specific estradiol binding sites. The binding profile obtained by incubating the cytosol with tritiated chloroquine and tritiated estradiol together tended to confirm the idea of separate binding sites. Scatchard analysis of the binding of chloroquine to uterine receptors showed the concentration of binding sites for chloroquine did not change in the presence of estradiol even though the dissociation constant increased by 16%. This also strengthens the concept of separate binding sites for estradiol and chloroquine.

Quinine, primaquine, paludrine and chloroquine all enhanced the binding of estradiol to rabbit uterine cytosol and in the case of chloroquine, it could be due to the binding of chloroquine itself to some proteins in the uterine cytosol.

Quinine is no longer the first choice drug for the treatment of malaria but the other three antimalarials are used.
Culture:

1 g of stool specimen was mixed with 9 ml of glycerol infusion broth and one loopful (2 mm) was cultured onto a set of two selective media: cycloserine, cefoxitin, fructose, blood agar (CCFA\(^{10}\)); a modification of CCFA; and cefoxitin, neomycin, fructose, blood agar (CNFA) developed as a substitute for primary isolation of \textit{Cl. difficile}. Each specimen was inoculated and streaked onto the media by a standard method that allowed semi-quantitative assessment of the density of growth on an arbitrary scale of 1\(^+\) - 5\(^+\). The culture plates were incubated anaerobically at 37 °C for 48 hours using the anaerobic generating kit system (Oxoid).

Yellow colonies on CCFA and CNFA resembling \textit{Cl. difficile} were picked and subcultured onto fresh Neomycin Blood agar and incubated at 37 °C for another 48 hours. Colonial morphology, smell, gram-stain appearance and yellowish green florescence on blood agar under ultra-violet light were used for the identification of isolates.

Clindamycin Serum Assay:

About 10 ml of venous blood was collected from each of the 36 volunteers on the 1st, and 5th days approximately 5 min before the next dose and 45 min after the last dose to determine the trough and peak clindamycin serum levels on each day. These levels were determined by standard methods\(^{11}\).

Adverse Reaction:

Adverse side effects were watched out for in all the volunteers who were given clindamycin. Assessment of side effects included monitoring skin rash and development of diarrhoea which was defined as loose bowel motions in excess of four times a day. The presence of cytotoxin produced by \textit{Cl. difficile} was not investigated because of technical difficulties.

Analysis of Data:

Student 't' test was used to analyse the results where appropriate.

Results

One hundred and seventy-seven (55\%) of the 324 routine faecal specimens yielded culture of \textit{Cl. difficile} with light to moderate growths (1\(^+\) - 3\(^+\)). Both CCFA and CNFA yielded the same number of positive cultures. In all, specimens from 178 males and 146 females were analysed. Eighty-Seven (48\%) of the 178 male specimens yielded \textit{Cl. difficile} while 90 (62\%) of the 146 female specimens were positive (p<0.05) (see table 1). The highest number of excretors were in the 21-30 age group.

Analysis of the specimens obtained from the 36 volunteers showed that 12 (33.3\%) were colonised by the \textit{Cl. difficile} prior to administration of clindamycin (table 2). By the 2nd day all of the 36 volunteers were culture-positive and remained so till after day 5. On the 5th, 10th and 20th days after stopping the clindamycin, 16, 21, and 22 volunteers respectively became culture-negative.

The range and mean (in brackets) trough and peak clindamycin serum levels were 0.45 - 2.9 (1.22) µg/ml and 3.8 - 5.9 (4.92) µg/ml on day 1, and 1.1-2.8 (1.23) µg/ml and 3.8 to 6.4 (4.80) µg/ml on day 5 respectively (table 3).

The semi-quantitative assessment of the density of growth of bacterial culture from the specimens obtained from the volunteers per day showed that the mean score on day 1 was 2.0\(^+\); range, 1\(^+\) - 3\(^+\). On day 2, through days 3, and 4 to day 5, the mean scores were 2.8\(^+\); range 1\(^+\) - 3\(^+\); 2.8\(^+\); range 1\(^+\) - 3\(^+\), 2.7\(^+\); range 1\(^+\) - 3\(^+\) and 2.8\(^+\), 1\(^+\) - 3\(^+\) respectively. There was no particular relationship between the clindamycin serum levels and the density of growth.

None of the 36 volunteers developed any objectively measurable side effect after administration of the clindamycin. No diarrhoea or PMS was observed.
### Table 1
Colonisation of adult gut by Cl. difficile in different age groups

<table>
<thead>
<tr>
<th>Faecal specimens</th>
<th>Number Cultured</th>
<th>Number (%) Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Male</td>
<td>178</td>
<td>87 (48)</td>
</tr>
<tr>
<td>Total Female</td>
<td>146</td>
<td>90 (62)</td>
</tr>
<tr>
<td><strong>Age groups (yrs).</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15 - 20</td>
<td>134</td>
<td>57 (43)</td>
</tr>
<tr>
<td>21 - 30</td>
<td>99</td>
<td>84 (85)</td>
</tr>
<tr>
<td>31 - 40</td>
<td>50</td>
<td>23 (46)</td>
</tr>
<tr>
<td>41 - 50</td>
<td>27</td>
<td>10 (37)</td>
</tr>
<tr>
<td>over 50</td>
<td>14</td>
<td>3 (21)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>324</td>
<td>177 (55)</td>
</tr>
</tbody>
</table>

### Table 2
Isolation of Cl. difficile from healthy volunteers after oral Clindamycin

<table>
<thead>
<tr>
<th>Duration of therapy</th>
<th>No of patients with culture - positive specimens</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>N = 36</strong></td>
<td></td>
</tr>
<tr>
<td>Pre-dose</td>
<td>12</td>
</tr>
<tr>
<td>Day 1</td>
<td>18</td>
</tr>
<tr>
<td>Day 2</td>
<td>36</td>
</tr>
<tr>
<td>Day 3</td>
<td>36</td>
</tr>
<tr>
<td>Day 4</td>
<td>36</td>
</tr>
<tr>
<td>Day 5</td>
<td>36</td>
</tr>
<tr>
<td>Post-cessation</td>
<td></td>
</tr>
<tr>
<td>Day 5</td>
<td>20</td>
</tr>
<tr>
<td>Day 10</td>
<td>15</td>
</tr>
<tr>
<td>Day 20</td>
<td>14</td>
</tr>
<tr>
<td>3 months after</td>
<td>14*</td>
</tr>
</tbody>
</table>

*Included the original 12 pre-dose positives.

### Table 3
Clindamycin serum levels of the volunteers

<table>
<thead>
<tr>
<th>Time of measurement</th>
<th>Clindamycin levels (µg/ml)</th>
<th>Trough</th>
<th>Peak</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>range</td>
<td>mean</td>
<td>range</td>
</tr>
<tr>
<td>Pre-dose</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Day 1</td>
<td>0.45 - 2.9</td>
<td>1.22</td>
<td>3.8 - 5.9</td>
</tr>
<tr>
<td>Day 5</td>
<td>1.1 - 2.8</td>
<td>1.23</td>
<td>3.8 - 6.4</td>
</tr>
</tbody>
</table>
Discussion

The results of this study amply demonstrate the common occurrence of *Clostridium difficile* in the faecal specimens of adult Nigerians. The *Clostridium difficile* isolation rate of 55% in routine specimens and 100% in specimens after clindamycin administrations showed that the organism is a very common member of the adult gut flora. This is in sharp contrast to the findings of other workers on its contribution to the normal flora of the gut of adult caucasians in Europe and America. No patient in the “routine” group had symptoms suggestive of *Clostridium difficile* infection and none of the volunteers had taken any antibiotics prior to the study. Both the initial isolation of *Clostridium difficile* in 33% of the 36 adult volunteers (pre-dose) and the isolation rate of 100% on the second day after clindamycin administration were remarkably high. This contrasts with the general belief that this organism is not a common flora of gastrointestinal tract of adults.

In this study majority of the colonised subjects (85%) were in the age group 21–30 years and most of them were females. The significance of this finding is not very clear especially when there was no concomitant *Clostridium difficile* associated infections in any of them. The role of *Clostridium difficile* without the production of cytotoxin therefore becomes less easily defined. Unfortunately the presence of cytotoxins in the faecal specimens was not investigated due to technical difficulties. The high isolation rate in volunteers prior to clindamycin administration further supports the results of routine specimen investigations and showed that the organism is indeed a common flora of the adult gut. The antibody levels (secretory and humoral) to *Clostridium difficile* and its cytotoxin in Nigerians is currently being investigated.

The results of the study on volunteers also confirmed that oral clindamycin did induce *Clostridium difficile* colonization of the adult gut. The relative increase in the density of growth between the base line semi-quantitative count and counts thereafter for five days indicated that the drug induced fairly heavy colonisation which was in line with the report of Kim et al.

It was worthy of note to find that the serum trough and peak levels of clindamycin in the volunteers had no relationship with the density of growth of *Clostridium difficile*. The mean peak level in our study was higher than the reported mean peak level in the American subjects, but without the development of pseudomembranous colitis (PMC) which, of course, confirms Bartlett’s report that the development of PMC has no relationship to either the dose or serum levels of the incriminated drug.

Clinical evaluation of the CNFA medium which is a modification of the well-tested and routinely used CCFA, by substitution of cycloserine with high concentration of neomycin (100 µg/ml), showed that the medium was just as sensitive as the CCFA for the primary isolation of *Clostridium difficile*.

All *Clostridium difficile* on the CNFA medium produced yellow and big colonies that flouresced golden yellow under ultra violet light. The density of growth was also comparable. Since cycloserine is not readily available in our country but neomycin is, its substitution by the latter should make the isolation of *Clostridium difficile* relatively easy in our laboratories. The medium should also be very useful for the screening of *Clostridium difficile*, in the busy routine laboratory, for large scale clinical work.

Acknowledgement

We thank Upjohn PTY Ltd., Lagos for providing clindamycin and Mr. Cyprian Tasie for excellent secretarial work.

References

1. Larson, H. E. and Price, A. B. Pseudo-