Short Communication

In vitro and In vivo interactions of methotrexate and other antimetabolites with the oestrogen high affinity receptors of the rat uterus

I.D. Morris & T.M. Stephen

Department of Pharmacology, Materia Medica and Therapeutics, Manchester University Medical School, Stopford Building, Manchester M13 9PT.

Breast tumour tissues of both animals and man contain a receptor system for oestrogen. Oestrogen combines with a high affinity receptor in the cytoplasm of the target cell and the complex is transferred to the nucleus from where the metabolic response of the tissue is directed (De Sombre, 1982; Jordan, 1982). In the past decade significant advances in the treatment of breast cancer were made after it was discovered that tumours could be classified as oestrogen-receptor (ER)-rich or ER-poor. This classification correlated with the response of the tumour during treatment by hormonal manipulation; ER-poor tumours did not respond whereas 50–60% of ER-rich tumours did (Henderson & Canellos, 1980; De Sombre, 1982). Thus the assay of tumour cytosols has become an important tool for the clinician to help in the assessment of treatment protocols. The drugs used for the treatment of breast cancer can be divided into 2 broad categories: One comprises the hormonally-related drugs which includes diethylstilboestrol and the antioestrogen tamoxifen. The other group includes an alkyating agent, cyclophosphamide, the antimetabolites, methotrexate and 5-fluorouracil and the antibiotic adriamycin, which are usually administered in the form of combined intermittent therapy (Henderson & Canellos, 1980). It is well established that the oestrogen agonists and antagonists will react with the ER system (Leclercq & Heuson, 1979). Recently it was demonstrated that 2 non-steroidal antimetabolites used for the treatment of breast cancer would also interact with the ER system of the rat uterus (Di Carlo et al., 1978). Methotrexate and adriamycin were shown to competitively prevent the binding of oestradiol-17β to the receptor in vitro. In vivo changes were also produced which suggested that these drugs reacted with the receptor system in a manner similar to oestrogen. These somewhat surprising results have important implications for breast cancer management. Treatment of a patient with these drugs prior to tumour biopsy could produce a false negative on a receptor screen or may even alter the response of a tumour to subsequent hormonal manipulation. However, if these drugs were acting via the ER system then a novel rationale for the use of receptor-directed cytotoxic drugs could be formulated with possibilities for improvement in the treatment of breast cancer. The antibiotic actinomycin D and cycloheximide have been used to investigate the role of protein synthesis in the establishment of tissue receptor levels (Cidlowski & Muldoon, 1978; Dix & Jordan, 1980; Horwitz & McGuire, 1980). As the results of Di Carlo et al. (1978) raised the possibility that they also may react directly with the receptor system, they have been included in this investigation, with tamoxifen and a variety of antimetabolites and antibiotics of markedly different molecular structures.

Immature female Sprague-Dawley rats (20–25 days old), bred in the Medical School, were used for the experiments. Drugs were administered i.v. via the tail vein dissolved in 0.2 ml of 0.9% w/v sodium chloride. Rats were killed by decapitation and the uterus dissected and rapidly cooled in ice cold buffer before weighing and homogenisation. [3H]-oestradiol-17β (Sp. Act. ~ 85 Ci mM⁻¹) was obtained from the Radiochemical Centre, Amersham, England. Oestradiol-17β, actinomycin D and cycloheximide were obtained from Sigma Chemicals (London) Ltd, Poole, Dorset, England. Methotrexate (powder-methotrexate USP. Potency 88.8% ampoules—methotrexate for injection 20 mg ml⁻¹), adriamycin (Doxrubicin hydrochloride) and tamoxifen were gifts from Lederle Laboratories, Gosport, Hampshire, Montedison Pharmaceutical Ltd, New Barnet, Hertfordshire and ICI Ltd, Macclesfield, Cheshire respectively. All other reagents were Analar grade.

Oestrogen receptor assay (a) in vitro experiments. Untreated rats were killed, the uteri removed and
homogenisation, cytosol preparation and the
determination of the number of oestrogen receptors
and their affinity for oestradiol-17β were carried
out as described by Ginsburg et al. (1974) with
the exception of the buffer system which is described
below. Uteri were homogenised in Tris-HCl
(0.01 M) buffer pH 7.4, containing EDTA 1 mM,
Dithiothreitol 0.5 mM. Homogenates were
centrifuged at 105,000 g for 1 h. Aliquots of the
supernatant fraction (cytosol) were incubated to
equilibrium at either 30°C (10 min) or 4°C (18 h)
with [3H]-oestradiol-17β (4 × 10⁻⁹ - 2 × 10⁻¹⁰ M)
with and without a 100 fold excess of
diethylstibestrol so that the oestrogen specific
binding could be determined. Non-radioactive
compounds, except methotrexate (10⁻³ M) and
tamoxifen, were added to the incubate dissolved in
10 μL of buffer to give the final concentration
required. In order to achieve the high concentration
of methotrexate (10⁻³ M), this agent was dissolved
in sodium hydroxide (0.1 N). The addition of 10 μL
of this solution to the incubate altered the pH from
7.4 to ~ pH 8, therefore a vehicle control was
included in the experiments. Low concentrations of
methotrexate and other drugs did not alter the pH
of the incubate. When methotrexate was prepared
from the commercially-prepared ampoules
concentrations > 10⁻⁶ M could not always be
achieved. Tamoxifen was added to the incubate
dissolved in ethanol, control incubates contained
ethanol alone. In experiments designed to examine
the effects of preincubation of the drug, the drug
was added to the cytosol 90 min or 10 min before
the [3H]-oestradiol for the 4°C or 30°C incubations
respectively. After incubation bound oestradiol was
separated from free oestradiol by the use of small
columns of Sephadex LH₂₀₀ maintained at 4°C. The
radioactivity in the eluate from this and other
experiments was determined by liquid scintillation
spectrometry. The effects of each series of the drugs
and their controls were determined upon one
cytosol and the results from several cytosols taken
to give the mean and s.e. (b) in vivo experiments.
Rats were injected with drug and, when
appropriate, 5 min later with oestradiol-17β; the
rats were killed after 1 h. A modified method of Roy
& McEwen (1977) was used to determine cytoplasmic
and nuclear oestrogen receptors (ERc;
ERN). The method was modified by the replacement
of the phosphate buffer with 0.1 M Tris-HCl buffer,
ph 7.6 and initially homogenising the uterus in
Tris-HCl buffer, ph 7.6 containing 0.32 M sucrose,
3 mM MgCl₂ and 0.5 mM dithiothreitol, so that a
high-speed cytosol could be prepared as outlined
above. ERₙ were prepared by extraction of purified
nuclei with Tris-HCl buffer ph 7.6 containing
0.4 M KCl, 0.5 mM dithiothreitol and 0.05 M
Bacitracin. Aliquots of the extract were incubated
with [3H]-oestradiol-17β (3 × 10⁻⁸ - 3 × 10⁻₁⁰ M)
for 2 h at 30°C with or without a 100-fold excess of
diethylstilboestrol to determine the oestrogen-
specific binding. Separation of bound from free
oestradiol was carried out as described previously.
The assayed concentrations of specifically-bound
oestradiol in the incubate were used to construct
plots (Scatchard, 1949) from which the equilibrium
dissociation constant Kᵣ and the saturation binding
capacity were determined. Binding of [3H]-
oestradiol-17β to the ERₙ is expressed per mg of
protein (the Lowry technique) or in terms of total
binding per uterus. The results are presented as the
mean ± s.e. and the significance of the difference
between groups calculated by Mann Whitney U test
(two-tailed).

Results

The effects of incubation of a variety of drugs with
the rat uterine ERₙ and [3H]-oestradiol are given in
the Table. Scatchard plots were constructed for all
incubation conditions except for tamoxifen 10⁻⁶ M
and methotrexate I (10⁻³ M, preincubation, 30°C)
when specific [3H]-oestradiol binding could only be
measured in one or two of the incubates. Apparent
dissociation constants for the reaction between
[3H]-oestradiol-17β and the uterine cytosol ranged
between 0.6 - 10 × 10⁻¹⁰ M. Methotrexate, prepared
from either ampoules or powder, incubated at 4°C
or 30°C, with or without preincubation did not
change the binding of [3H]-oestradiol to the cytosol
in concentrations up to 10⁻⁶ M. Methotrexate at
10⁻³ M decreased the [3H]-oestradiol binding when
compared to vehicle; in general, the changes were
small and not always significant (see Table).

Actinomycin D, adriamycin and cycloheximide
did not alter [3H]-oestradiol binding when added
to the incubates in concentrations up to 10⁻⁶ M. In
contrast tamoxifen (10⁻⁶ M) almost completely
inhibited the specific binding of [3H]-oestradiol to
the uterine cytosol.

Methotrexate and adriamycin when administered
to the rat by i.v. injection did not alter the pattern
of ER distribution in the uterus, oestradiol-17β
decreased the ERₙ and increased the ERₙ content of
the uterus (Figure). When methotrexate was
administered to the rats at the same time as
oestradiol-17β the uterine cytosol receptor content
was not changed; the nuclear receptor content
appeared to decrease but this change did not
achieve significance (P > 0.05). The receptor
distribution after oestradiol and adriamycin was
similar to the distribution found after oestrogen
alone.
Table I The in vitro effects of some non steroidal drugs upon the specific binding of [3H]-oestradiol to cytosol prepared from immature rat uteri. The interactions were examined at 4 or 30°C with or without preincubation as described in the methods. The methotrexate used for the experiments, Methotrexate I and Methotrexate II, was supplied as a powder or in commercially prepared ampoules respectively. V = alkaline vehicle control. The results (mean ± s.e.m., n = 4–8) are expressed as the number of binding sites per mg cytosol protein \times 10^{11}.

| Drug concentration (M) | 0      | 10^{-9} | 10^{-6} | 10^{-3} | V       |
|------------------------|--------|---------|---------|---------|---------|
| **Methotrexate I**     |        |         |         |         |         |
| 4°C                    | 5.8±0.4| 6.4±0.3 | 5.8±0.3 | 5.3±0.4 | 6.0±0.3 |
| 30°C                   | 4.6±1.4| 5.6±1.6 | 5.0±1.6 | 2.9±1.3 | 5.0±1.5 |
| Preincubation          |        |         |         |         |         |
| 4°C                    | 6.1±0.4| 5.9±0.4 | 6.2±0.6 | 5.1±0.3 | 5.9±0.3 |
| 30°C                   | 3.6±0.7| 3.9±0.6 | 3.5±0.4 | 1.2*    | 3.9±0.7 |
| **Methotrexate II**    |        |         |         |         |         |
| 4°C                    | 6.0±0.3| 5.8±0.2 | 6.1±0.2 | 5.2±0.4 | ND      |
| Preincubation          |        |         |         |         |         |
| 4°C                    | 5.7±0.8| 5.2±0.7 | 5.2±0.6 | ND      | ND      |

| Concentration (M)      |        |         |         |
|------------------------|--------|---------|
| **Adriamycin**         |        |         |
| 4°C                    | 9.1±0.5| 8.7±0.7 | 10.9±1.9|
| Preincubation          |        |         |
| 4°C                    | 5.0±0.7| 5.8±0.7 | 7.8±0.7 |
| **Actinomycin D**      |        |         |
| 4°C                    | 3.4±0.4| 3.7±0.5 | 3.7±0.4 |
| **Cycloheximide**      |        |         |
| 4°C                    | 5.7±1.3| 5.1±1.2 | 5.5±1.22|
| **Tamoxifen**          |        |         |
| 4°C                    | 13.7±3.7| 10.1±2.8| 5.5*    |
| 30°C                   | 3.8±1.0| 3.6±0.6 | 0.4*    |

ND = not determined.

*Only 1 or 2 cytosols gave results which could be distinguished from diethylstilboestrol incubates.

Significance of differences from appropriate control values *P < 0.05.

The in vitro and in vivo experiments suggests that the antibiotics actinomycin D and adriamycin, & the antimetabolites cycloheximide and methotrexate do not specifically react with the oestrogen receptors. These results are in agreement with those of Muller et al. (1980) and fail to confirm the data of Di Carlo et al. (1978). Muller et al. (1980) assayed oestrogen binding by saturation analysis, employing a single oestradiol concentration, whereas Di Carlo et al. (1978) used multiple oestradiol concentrations and Scatchard (1949) analysis which might have accounted for the different results. However, our experiments examined the binding of oestradiol-17β under a variety of rigidly-controlled conditions (single and multiple oestradiol-17β concentration saturation analysis, co- and pre-incubation of the competitor and incubation at either 4°C or 30°C), yet we could not demonstrate the high level of competition that was shown by Di Carlo et al. (1978). Significant changes in oestradiol-17β binding were found only after incubation with very high concentrations of
methotrexate \(\left(10^{-3}\ M\right)\), therefore it is possible that the inhibition of binding by these drugs of substantially different molecular structure from the steroids, is a result of non-specific interaction. A specific interaction was shown by the use of tamoxifen which is well known to possess high affinity for the ER (Dix & Jordan, 1980; Jordan, 1982). In this case a substantial reduction in the binding of oestradiol-17\(\beta\) to the receptor was demonstrated.

The actions of oestrogens upon the target cell is associated with a decrease in the ER, concentration as the receptor ligand complex is translocated to the nucleus. This response is well documented (see for example Dix & Jordan, 1980) and has been demonstrated in the present series of experiments. Similar changes may have been predicted if adriamycin and methotrexate reacted directly with the receptor, yet none were seen. If the reaction with the receptor was in some way not detectable by the current methods then these drugs, when administered with oestradiol, would be expected to alter the pattern of receptor changes. Once again no change was demonstrated. These data confirm the conclusions from the \textit{in vitro} experiments that these drugs do not react directly with ER.

Actinomycin D and cycloheximide were without effect upon the \textit{in vitro} binding of oestradiol-17\(\beta\) to the receptor, so it is reasonable to assume that the receptor changes reported in previous papers (Cidlowski & Muldoon, 1978; Dix & Jordan, 1980; Horwitz & McGuire, 1980) reflects the role of protein synthesis in the maintenance of tissue H.A.R. concentrations. Such experiments have led to the conclusion that receptor concentrations are maintained by 2 mechanisms, \textit{de novo} synthesis and recycling. Methotrexate, adriamycin and many other drugs used for the treatment of breast cancer will inhibit protein synthesis, so that while the present experiments have failed to demonstrate a direct effect of these drugs upon ER an indirect action \textit{via} inhibition of receptor protein synthesis cannot be discounted.

Figure 1  The uterine content of cytosol (open bars) and nuclear (closed bars) ER determined \textit{in vitro}. The uterine fractions were prepared from rats killed 1 h after the i.v. injection of saline (C, 0.2 ml), methotrexate (M, 1 mg kg\(^{-1}\)), adriamycin (A, 0.5 mg kg\(^{-1}\)), oestradiol-17\(\beta\) (E, 5 \(\mu\)g) or combinations of these drugs (Mean ± s.e., n = 4-7).

We are grateful to the Cancer Research Campaign for financial support and to Lederle Laboratories, Montedison Pharmaceuticals Ltd and ICI Ltd for their gifts of drugs.
References

CIDLOWSKI, J.A. & MULDOON, T.G. (1978). The dynamics of intracellular estrogen receptor regulation as influenced by 17β estradiol. Biol. Reprod., 18, 234–246.
DE SOMBRE, E.R. (1982). Breast cancer: hormone receptors, prognosis and therapy. Clinics in Oncology, 1, 191–214.
DI CARLO, F., REBOANI, C., CONTI, C. & GENAZZANI, E. (1978). Changes in the concentration of uterine cytoplasmic oestrogen receptors induced by doxorubicin and methotrexate. J. Endocrinol., 79, 201–208.
DIX, C.J. & JORDAN, V.C. (1980). Modulation of rat uterine steroid hormone receptors by estrogen and antiestrogen. Endocrinology, 107, 2011–2020.
GINSBURG, M., GREENSTEIN, B.D., MACLUSKY, N.J., MORRIS, I.D. & THOMAS, P.J. (1974). An improved method for the study of high affinity steroid binding: oestradiol binding in brain and pituitary. Steroids, 23, 773–792.
HENDERSON, I.C. & CANELLOS, G.P. (1980). Cancer of the breast. N. Engl. J. Med., 302, 17–30, 78–90.
HORWITZ, K.B. & MCGUIRE, W.L. (1980). Nuclear estrogen receptors, effect of inhibitors on processing and steady state levels. J. Biol. Chem., 255, 9699–9705.
JORDAN, V.C. (1981). Laboratory models of hormone-dependent cancer. Clinics in Oncology, 1, 21–40.
LECLERCQ, G. & HEUSON, J.C. (1979). Physiological and pharmacological effects of estrogens in breast cancer. Biochim. Biophys. Acta, 560, 427–455.
MÜLLER, R.E., SHEARD, B.E., TRAISH, A. & WOTIZ, H.H. (1980). Effect of chemotherapeutic agents on the formation of estrogen-receptor complex in human breast tumour cytosol. Cancer Res., 40, 2941–2942.
ROY, E.J. & MCEWEN, B.S. (1977). An exchange assay for estrogen receptors in cell nuclei of the adult rat brain. Steroids, 30, 657–669.
SCATCHARD, G. (1949). The attractions of proteins for small molecules and ions. Ann. N.Y. Acad. Sci., 51, 660–672.