J. steroid Biochem. Vol. 23, No. 6A, pp. 949–953, 1985
Printed in Great Britain.

0022-4731/85 $3.00 + 0.00

EFFECTS OF MOLYBDATE ON CLASSIFICATION OF ESTROGEN AND PROGESTERONE RECEPTOR STATUS IN HUMAN BREAST CANCER

BJORN W. BORJESSON* and CHRISTOPHER J. TESORIERO
The New South Wales State Cancer Council, Special Unit for Investigation and Treatment, Prince of Wales Hospital, Randwick, NSW 2031, Australia

(*Received* 27 *July* 1984)

Summary—Incorporation of disodium molybdate in the homogenization buffer for breast cancer specimens was shown to inhibit a time and temperature dependent loss of estrogen (ER) and progesterone (PR) receptor binding capacity in the cytosols. Since positive receptor values are correlated with response to endocrine therapy, the effect of molybdate incorporation on ER/PR classification was further investigated in two separate series of specimens. In one series of 75 consecutive specimens, ER and PR were analyzed before incorporation of molybdate in the asssays, and the results compared with the next 75 consecutive specimens after incorporation of molybdate. The mean ages of the 2 groups of patients were not significantly different (59.1 ± 13.4 versus 59.4 ± 13.3 years). It was found that the PR binding capacity was significantly higher, the proportion of ER+/PR− specimens was significantly reduced, and that a bimodal distribution of ER+ specimens only became apparent when molybdate was incorporated in the ER and PR assays. The possibility exists that ER+ specimens with binding capacities above 175 fmol/mg protein, constituting 24% of all the specimens and representing the second bimodal peak, may represent hormone dependent tumours.

INTRODUCTION

Both estrogen (ER) and progesterone (PR) receptor status is important for predicting response to endocrine therapy in breast cancer [1, 2]. However, it has been suggested that the proportion of breast carcinomas that is PR positive has been underestimated in previous work due to the high susceptibility of PR to inactivation when molybdate has not been incorporated in the assay buffer [3]. Furthermore, although ER is less susceptible to inactivation, molybdate incorporation may also raise the apparent ER values in some breast carcinomas [3], or at least stabilize the high molecular forms of ER [4].

Recent studies with female reproductive tract tissues [5], have recommended that molybdate should be added to the homogenization buffer and not only to the prepared cytosol as in previous studies [3, 4], in order to minimize loss of receptor binding capacity during cytosol preparation.

In the present study, molybdate was therefore added to the homogenization buffer, and the loss of ER and PR binding capacity with time and temperature was investigated. In addition, the effects of molybdate incorporation on the classification of ER/PR status were examined as well as its effect on the quantitative correlation between ER and PR values.

*To whom correspondence should be addressed.
Present address: Garvan Institute of Medical Research, St Vincent's Hospital, Darlinghurst, NSW 2010, Australia.

EXPERIMENTAL

Isotopes and chemicals

[2,4,6,7-^{3}H]1,3,5(10)-Estratrien-3,17β-diol(^{3}HE$_2$, 89 Ci/mmol), 16α-Ethyl-21-hydroxy-19-nor[6,7-^{3}H]-pregn-4-ene-3,20-dione (^{3}H Org-2058, 48 Ci/mmol) and 16α-ethyl-21-hydroxy-19-norpregn-4-ene-3,20-dione (Org-2058) were purchased from Amersham (Sydney, Australia). Diethylstilbestrol (DES) was purchased from Sigma Chemical Co. (St Louis, U.S.A.), and human serum albumin (HSA) was purchased from Calbiochem-Behring (San Diego, U.S.A.). All other chemicals were reagent grade.

Buffers

Cytosols were prepared using a buffer consisting of 10 mM Tris, 1.5 mM EDTA, 10% v/v glycerol and 1 mM monothioglycerol adjusted to pH 7.4 with 1 M HCl, with and without 10 mM disodium molybdate. Radioactive steroids and competitors were dissolved in these buffers, but monothioglycerol was then omitted.

Tissue handling and storage

Breast cancer specimens from a number of sources were stored at −70°C for less than 2 weeks prior to ER and PR assays. Any residual tissue from receptor positive specimens was pooled and stored at −70°C for up to 3 months.

Cytosol preparation

Tissue specimens were fragmented by percussion

between two steel blocks prior to pulverization in a Microdismembrator II (B. Braun Melsungen AG, West Germany) after snap freezing in liquid nitrogen. Cytosols were prepared using a tissue to buffer ratio of 1:6 (w/v), and centrifugation at 67,000 *g* for 1 h at 4°C.

Receptor assays

The assays were performed as previously reported [6], but with the modification that [^{3}H]Org-2058 ± 200 × excess Org-2028 was used to determine PR. The receptor analysis was also modified in that the linearity was determined by Scatchard plot analysis and the receptor concentrations and dissociation constants (K_d) calculated by the Woolf plot. The protein concentrations were determined by the method of Lowry [7] using a HSA standard.

RESULTS

Molybdate, time, temperature and ER/PR binding

The effect of molybdate on the loss of PR binding capacity in cytosols with time and temperature for samples taken from a pool of 15 breast cancer specimens is shown in Fig. 1. It can be seen that the addition of molybdate prevented loss of binding capacity at both 4 and 26°C for at least 5 h. In the absence of molybdate, PR was not measurable in these samples (with the exception of 1 sample) at either temperatures (Fig. 1).

The effect of molybdate on loss of ER binding capacity is also shown in Fig. 1. No significant change

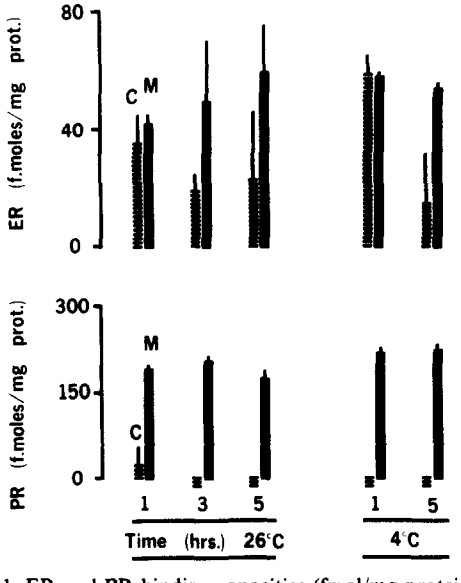

Fig. 1. ER and PR binding capacities (fmol/mg protein) in pooled human breast cancer cytosols are shown as a function of incubation time and temperature in control buffer (C) without 10 mM molybdate, and in molybdate buffer (M) in the absence of ligands. The cytosols were left standing for the given times and temperatures, and then assayed for ER and PR. The values represent the mean and range of independent duplicates.

Table 1. The effect of molybdate on ER and PR binding capacities in the cytosols from one human breast cancer specimen

Aliquot	Molybdate	ER	PR
1	+	105	288
2	+	102	281
3	–	79	202
4	–	84	182
% Decrease		21	33

in ER binding capacity with incubation time and temperature was observed in the presence of molybdate. By contrast, a decrease in binding capacity with time at both 4 and 26°C was observed in the absence of molybdate, and two samples were classified as ER negative after 5 h incubation (Fig. 1).

A sufficiently large breast cancer specimen received for routine ER/PR assays made it possible to investigate the effects of molybdate on a single specimen rather than on pooled specimens. The specimen was pulverized and divided into 4 aliquots, and cytosols were prepared with and without molybdate incorporation. The results of the routine assays at 4°C are shown in Table 1. It can be seen that the binding capacity obtained when molybdate had not been incorporated were 21% lower for ER and 33% lower for PR.

Molybdate and ER/PR status

The ER and PR binding capacities in primary breast cancer specimens from 75 consecutive patients were measured in the absence of molybdate and compared with the measurements in specimens from the next 75 consecutive patients. Assay methods (with the exception of molybdate incorporation) were not changed during the study, and all the assays were performed by the same experienced operator. The mean age of the patients in the first group (minus molybdate) was 59.1 ± 13.4 (SD) years and not significantly different from the mean age of the second group (plus molybdate) which was 59.4 ± 13.3 SD) years.

The effect of molybdate incorporation on ER/PR status is shown in Table 2. It can be seen that in the absence of molybdate, the distribution of ER/PR status was similar to that previously reported [1]. However, when molybdate was incorporated (Table 2), the proportion of ER+/PR+ specimens increased significantly (Chi-square test) from 34.7 to 53.3% ($P = 0.032$), while the proportion of ER+/PR– specimens decreased significantly from 22.7 to 9.3% ($P = 0.043$). By contrast, the proportion of ER+ specimens (ER+/PR+ and ER+/PR–) did not change significantly ($P = 0.62$) when molybdate was incorporated in the assays.

Molybdate and ER/PR correlations

The binding capacities of ER and PR are compared for specimens positive for either ER, PR on both in Fig. 2. No significant correlation between the

Table 2. Distribution of ER and PR status in human breast cancer

	ER+/PR+	ER+/PR−	ER−/PR+	ER−/PR−
Lit.($n = 638$)*	41.2%	29.6%	2.4%	26.8%
−Mo($n = 75$)	34.7%	22.7%	6.7%	36.0%
+Mo($n = 75$)	53.3%†	9.3%†	5.3%	32.0%

*Literature review [1] of ER and PR distribution.
†Significant differences ($P < 0.05$) in ER and PR distribution before and after introduction of molybdate (Mo) to the homogenization buffer.

ER and PR binding capacities was observed when molybdate had not been incorporated in the assays ($P = 0.074$, Spearman Rank test). When molybdate had been incorporated, the correlation between ER and PR binding capacities improved and was significant at the 0.05 level ($P = 0.026$). However, substantial scattering in the correlations between ER and PR binding capacities was observed (Fig. 2). The median ER binding capacity in Fig. 2 increased 36% from 50 to 68 fmol/mg protein, and the median PR binding capacity increased 133% from 21 to 49 fmol/mg protein when molybdate had been incorporated in the assays. The increase in ER binding capacity was not significant ($P = 0.34$), whereas the increase in PR binding capacity was significant ($P = 0.012$) as determined by the Wilcoxon test.

Molybdate and ER/PR frequency distributions

The effect of molybdate on the frequency distributions of ER and PR binding capacities is shown in Fig. 3. It can be seen that for the limited number of specimens assayed, a bimodal distribution was obtained for the ER+ specimens when molybdate had been incorporated in the assay. The second peak above 175 fmol/mg protein represented 24% of the 75 specimens analysed for ER. No bimodality was apparent for ER binding capacities when molybdate had not been incorporated, or for the PR values irrespective of molybdate incorporation (Fig. 3).

DISCUSSION

Experimental design

It is essential for a "historical" design as in the present study, that the two groups of patients and measurements are identical in all respects with the only exception being the parameter which is investigated, in this case the incorporation of molybdate in the ER/PR assays. These conditions were satisfied by including a sufficient number of patients in two consecutive groups (75 patients in each) as shown by the insignificant age difference between the groups (59.1 ± 13.4 versus 59.4 ± 13.3 years) and by changing only one parameter in the assays (molybdate incorporation) which were performed by only one experienced operator on a routine basis.

The possibility that improved operator skills during the second part of this study resulted in higher ER and PR values when molybdates were introduced is unlikely for 3 reasons. Firstly, the operator had performed more than 100 assays prior to commencing the present study. Secondly, the effect of molyb-

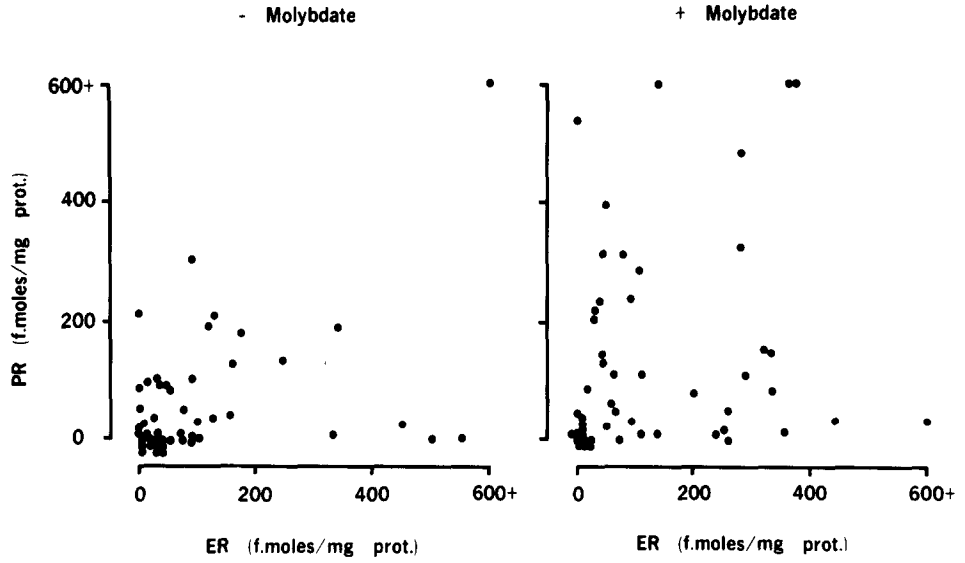

Fig. 2. Comparison of ER and PR binding capacities (fmol/mg protein) in receptor positive human breast cancer specimens. The binding capacities were compared for 48 specimens that were positive (> 1 fmol/mg protein) for either ER, PR or both when molybdate had not been included in the assays (−Molybdate) and for 51 specimens that were positive when molybdate had been incorporated (+Molybdate).

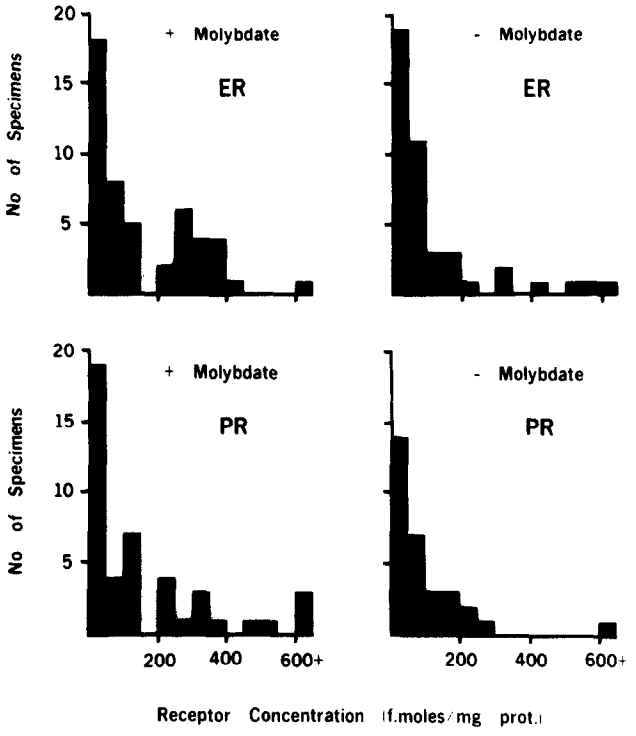

Fig. 3. Frequency distributions of the number of specimens within the given ranges of receptor binding capacities (1 to 50, 51 to 100, etc) for ER and PR with molybdate omitted (−) or incorporated (+) in the assays.

date is demonstrated under identical conditions (± molybdate) in two separate experiments (Table 1 and Fig. 1). Thirdly, the effect of molybdate is compatible with the literature cited. The pitfalls of an "historical design" have therefore carefully been avoided in the present study.

Molybdate, time, temperature and ER/PR binding

It was found that when molybdate had not been incorporated, the ER and PR binding capacities in human breast cancer showed a time and temperature dependent decrease which was most pronounced for PR, and that individual specimens may differ in their susceptibility to loss of binding capacity (Table 1 and Fig. 1). These results clearly demonstrate that for human breast cancer specimens, molybdate should be incorporated in the homogenization buffer and not only to the prepared cytosols [3, 4] in order to prevent loss of ER and PR binding capacities during cytosol preparation.

Molybdate and ER/PR status

The difference in susceptibility between ER and PR to loss of binding capacity when molybdate is not incorporated, is reflected in the qualitative results shown in Table 2. Molybdate had no significant effect on the proportion of ER+ specimens (Table 2), although the quantitative ER values increased 36% when it had been incorporated. In contrast, the increase for the quantitative PR values was 133%, and resulted in a significant increase in the proportion of PR+ specimens when molybdate had been incorporated (Table 2). These observations support the contention that for specimens with initially low PR values, the PR content may not be measurable if molybdate has not been incorporated, resulting in a high proportion of ER+/PR− specimens and a decrease in the proportion of PR+ specimens.

Molybdate and ER/PR correlations

It has previously been shown for MCF-7 human breast cancer cells that PR synthesis is ER dependent [2]. It was therefore interesting to note that when loss of the PR binding capacity was minimized by molybdate incorporation, the correlation between the ER and PR values improved from $P = 0.074$ to $P = 0.026$ (Fig. 2). Taken in conjunction with the reduced proportion of ER+/PR− specimens and increased proportion of ER+/PR+ specimens when molybdate has been used to minimize loss of binding capacity (Table 2), these results suggest that for a majority of breast carcinomas, ER and PR synthesis may be closely associated with each other.

Molybdate and ER/PR frequency distributions

It is now well established that high ER values correlate with improved remission rates in human breast cancer [8]. The biomodal distribution of ER values shown in Fig. 3, where 24% of the specimens investigated had ER values greater than 175 fmol/mg protein, may therefore be of particular interest. Although the amount of data is limited, they suggest the

possibility of improved correlation between ER values and remission rates in breast cancer when molybdate is used to minimize loss of binding capacity. However, clinical data is needed to fully evaluate this possibility. It should also be noted that from previously published data [9, 10], it can be shown that when molybdate is added to the prepared cytosol, no bimodality is observed for the ER values. These observations do not exclude the possibility of improved correlation between ER values and remission rates, especially when molybdate is also incorporated in the homogenization buffer in order to prevent loss of binding capacity during the whole cytosol preparation procedure.

CONCLUSIONS

When molybdate was incorporated during the whole procedure, it was observed that the proportion of PR+ specimens increased, the proportion of ER+/PR− specimens decreased, the proportion of ER+ specimens remained unchanged, the PR values increased significantly in absolute terms, the increase in ER values was not significant, the correlation between ER and PR values improved, and a bimodal distribution of ER values was obtained. It is concluded that molybdate incorporation is essential to prevent loss of binding capacity during the whole ER and PR assay procedure for human breast cancer, and that clinical trials should commence to establish whether an improved correlation between ER/PR values and remission rates following endocrine treatment can be achieved by molybdate incorporation.

Acknowledgements—The statistical and technical assistance of Dr Richard Fisher and Mrs Helen Vosnakis respectively, were very much appreciated.

REFERENCES

1. McGuire W. L. and Clark G. M.: Progesterone receptors and human breast cancer; "Wassink Lecture" presented at the 3rd EORTC breast cancer working conference. *Eur. J. Cancer clin. Oncol.* **19** (1983) 1681–1685.
2. Horwitz K. B. and McGuire W. L.: Estrogen control of progesterone receptor in human breast cancer. *J. biol. Chem.* **253** (1978) 2223–2228.
3. Anderson K. M., Phelan J., Marogil M., Hendrickson C. and Economou S.: Sodium molybdate increases the amount of progesterone and estrogen receptors detected in certain human breast cancer cytosols. *Steroids* **35** (1980) 273–280.
4. Miller L. K., Tuazon F. B., Niu E. and Sherman M. R.: Human breast tumour estrogen receptor: effects of molybdate and electrophoretic analysis. *Endocrinology* **108** (1981) 1369–1378.
5. Toppila M., Willcocks D., Tyler J. P. P., Eastman C. J. and Hudson C. N.: The effects of storage time and molybdate on steroid receptors in gynaecological tissues. *Steroids* **40** (1982) 139–148.
6. Keightley D. D. and Cressie N. A. C.: The Woolf plot is more reliable than the Scatchard plot in analysing data from hormone receptor assays. *J. steroid Biochem.* **13** (1980) 1317–1323.
7. Lowry, O. H., Rosebrough N. J., Farr A. L. and Randall R. J.: Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193** (1951) 265–265.
8. McGuire W. L.: Steroid hormone receptors in breast cancer treatment strategy. *Recent Prog. Horm. Res.* **36** (1980) 135–156.
9. Anderson K. M., Bonomi P., Morogil M., Henrickson C. and Economou S.: Comparison of dextran-coated charcoal and sucrose density gradient analysis of estrogen and progesterone receptors in human breast cancer. *Cancer Res.* **40** (1980) 4127–4132.
10. Lazier C. B., Richman J. and Longergan K.: The effect of molybdate on the intercellular distribution of estrogen receptor in mammary tumours. *Breast Cancer Res. Treat.* **4** (1984) 19–26.