Pathol. Oncol. Res. (2009) 15:115–121
DOI 10.1007/s12253-008-9117-x

BRIEF COMMUNICATION

N-methylnitrosourea Induced Breast Cancer in Rat, the Histopathology of the Resulting Tumours and its Drawbacks as a Model

M. Perše · A. Cerar · R. Injac · B. Štrukelj

Received: 14 March 2008 / Accepted: 16 October 2008 / Published online: 5 November 2008

Abstract Several animal models of breast cancer have been developed to study various aspects of breast cancer biology. Substantial evidence suggests that the *N*-methylnitrosourea (MNU) animal model mimics human breast cancer in many respects. It has therefore been used extensively to evaluate preventive and therapeutic agents for human breast cancer. Chemically induced rodent models are also suitable for studying malignant progression. Recently, Liska et al. [7] established two protocols of MNU administration depending on the animal's age and number of applications of carcinogen, with the aim of investigating the advanced stages of mammary gland tumours. We used the same protocol as Liska but have obtained substantially different results. These results are presented and discussed in the frame of suggested key drawbacks of the MNU induced breast cancer rat model, as a contribution to the debate about the suitability of that model for evaluating preventive and therapeutic agents.

Keywords Animal model · Breast cancer · Histopathology · *N*-methylnitrosourea · Rats

M. Perše (✉) · A. Cerar
Institute of Pathology, Medical Experimental Centre,
Medical Faculty, University of Ljubljana,
Korytkova 2,
1105 Ljubljana, Slovenia
e-mail: martina.perse@mf.uni-lj.si

R. Injac · B. Štrukelj
Faculty of Pharmacy, Institute of Pharmaceutical Biology,
University of Ljubljana,
Askerceva 7,
1000 Ljubljana, Slovenia

Introduction

Breast cancer is the most frequent malignancy diagnosed in women worldwide [1]. Several animal models of breast cancer have been developed to study varied aspects of breast cancer biology (described in [2]).

Two of them, that are also the most frequently used for the study of rat mammary tumorigenesis, are ones in which tumours are induced in the Sprague Dawley rat by 1,2-dimethylbenz (a)-anthracene (DMBA) or by *N*-methylnitrosourea (NMU; MNU) [3]. MNU is a highly specific carcinogen for the mammary gland and, in contrast to DMBA, does not require metabolic activation. MNU induced mammary tumours are also locally aggressive and able to metastasize, while the DMBA induced tumours are not [4].

It has been shown that the tumour incidence, latent period and tumour multiplicity are strain [5], dose and age [4–6] dependent, irrespective of the route of administration [6]. There are several protocols, involving time of carcinogen instillation, dose and routes of carcinogen applications, for inducing mammary tumours in Sprague Dawley rats [7–9]. We used the one introduced by Liska and co-workers [7] but our results differed from those of Liska. We present these results, together with the characteristics of the tumours, and indicate the drawbacks of the model. However, from the point of animal welfare, humane endpoints, ethic considerations and scientific results it is very important to have enough information about the chosen model to design experiment responsibly and to evaluate results properly.

Materials and Methods

Animals

The study was conducted in accordance with the European Union legislation on animal experimentation. Thirty-nine outbreed female Sprague Dawley rats were obtained from Harlan, Italy at 3 weeks of age. The animals had free access to standard rodent pelleted diet (Altromin, Germany) and tap water. They were kept in an animal room with 22–23°C air temperature and 55±10% humidity with a 12 h light-dark cycle (6.00–18.00 light). The care of laboratory animals followed accepted standards in European Union.

Carcinogen

Mammary tumours were induced by MNU (Sigma Chemical Co., St. Louis, MO) prepared according to the method described [6]. MNU was dissolved immediately before use in 0.9% NaCl and adjusted with acetic acid to pH value 4. Fresh solutions were prepared prior to application.

Experimental Design

After quarantine, 39 animals were randomly divided and housed 3 to 4 per cage. At 7 and 16 weeks of age all 32 experimental rats were given MNU intraperitoneally at a dose of 50 mg/kg body weight [7]. The seven control rats at the same ages received sterile saline only. Rats were weighed weekly. After 3 weeks of the first MNU application they were palpated twice a week for the presence of mammary tumour. When a tumour was first palpated, the date and its location were recorded. The experiment was terminated by euthanasia at 14 weeks after the first MNU application, when complete autopsy was performed. The experiment was carried out from January to May. All procedures on animals were executed between 8.00 and 11.00 h in the morning.

Morphological Techniques

Rats were euthanized by CO_2 asphyxiation and their mammary glands evaluated for the presence of tumours. Immediately after euthanasia, blood from the right ventricle was obtained for haematological examination (Table 1). The lungs, liver, spleen, kidney and lymph nodes were removed and examined for metastasis. All palpable tumours were excised, weighed and their size and location recorded. Palpable and microscopic tumours and suspicious lesions were fixed in 10% buffered formalin and later embedded in paraffin, sectioned and stained with hematoxylin-eosin for histological evaluation. Mammary tumours were classified histologically according to the criteria outlined by Russo [10]. The histopathological criteria used to determine malignancy were loss of tubular-alveolar pattern of the normal mammary gland, presence of large epithelial cells with increased nuclear-cytoplasmic ratio and increased frequency of mitosis, stromal response by fibrosis and inflammatory cell infiltration, necrosis and haemorrhage, and evidence of infiltration of surrounding tissues and metastasis.

Table 1 Haematological results of MNU and control groups

	Control group	MNU group
RBC (x 10^{12}/L)	6.7±2.1	5.1±1.7
Hb (g/dL)	13.2±4.6	11.0±3.3
Ht (%)	37.7±12.3	31.9±9.8
MCHC (g/dL)	36.4±12.0	34.7±0.5
WBC (x 10^9/L)	6.9±2.4	14.1±5.3
Neutrophil (%)	8.7±6.5	17.0±3.2*

*Represents significant difference from the control ($p<0.05$)

Statistical Evaluation

Results are expressed as means + SEM. Data was analyzed by independent samples t-test.

Results

Haematological Results

The values of red blood cells, haemoglobin, haematocrit and MCHC were slightly lower in the MNU treated group than in the control group, indicating a tendency to anaemia. On the other hand the number of white blood cells was considerable increased. The number of neutrophils was also significantly higher in the MNU group, indicating an inflammatory response in animals with large tumours (Table 1).

Rat Growth

Administration of MNU induced no acute toxicity in treated rats. No significant differences were observed in the body weight gain between the groups. However, 1 week after the second MNU application, a slightly smaller increase in body weight was observed in the MNU group than in the control group. Otherwise, the body weight of all rats increased up to the end of the experiment. 14 weeks after the first MNU application one rat became moribund and was humanely euthanized. Its weight loss was 6 g (2.4% of its body weight) due to its large mammary tumour, which ulcerated the skin.

Tumour Incidence and Multiplicity

The first palpable mammary tumours appeared 9 weeks after the first application of MNU. At this time only 2 of the 32 rats developed grossly detectable tumours. Two weeks later 19 rats (59 %) had palpable tumours. Tumour incidence increased with time and, at the end of the experiment, 30 rats (93.7%) had grossly detectable mammary tumours (Fig. 1).

Histopathological examination revealed 100% mammary tumour incidence. All 158 tumours were of mammary origin. The number of tumours per rat was 4.94±0.573 (range from 1 to 15 tumours). The majority of rats (71.8%) developed 2 to 6 mammary tumours each (Fig. 2).

At autopsy the clitoral glands of 2 rats were enlarged. Histological examination revealed hyperplasia in one and squamous papilloma in the other (Fig. 3). Sometimes, together with inguinal mammary tumours, part of the clitoral gland tissue was also excised, and was diagnosed as being histologically normal.

Tumour Weight

The mean weight of grossly detectable mammary tumours was 8.17±1.52 g (range from 0.03 g to 16.9 g per tumour). The weight of total mammary tumours per rat (total tumour mass) ranged from 1.0 g to 33.65 g. The majority of rats (59%) had a total tumour mass lower than 6.0 g per rat (less than 2% of body weight), but the total tumour mass of 9 rats (28%) ranged from 8.0 g to 18.53 g per rat (3–7% of body weight) and the mass of 3 rats (9.4%) ranged from 21.0 g to 27.0 g per rat (7.9–10% of body weight). Furthermore, one of the rats developed multiple tumours of total mass 33.65 g, (12.6% of their body weight).

Location of Tumours

MNU administration resulted in the induction of an average of 1.9 times as many mammary carcinomas in the cervical and thoracic mammary glands as in the abdominal and inguinal glands. The weights of the grossly detectable tumours in the cervical and thoracic mammary glands were of an average of 2.5 times greater than in the abdominal and inguinal glands and exhibited more invasive character. Differences were also observed in cancer occurrence in the right and left mammary gland chains (ratio 1.4:1). The difference was due to the larger number of the cervical-thoracic mammary gland tumours. The ratio of the weights of the right to the left mammary gland tumours was even greater (ratio 2.3:1), but this time due to the larger weight of the abdominal-inguinal mammary gland tumours.

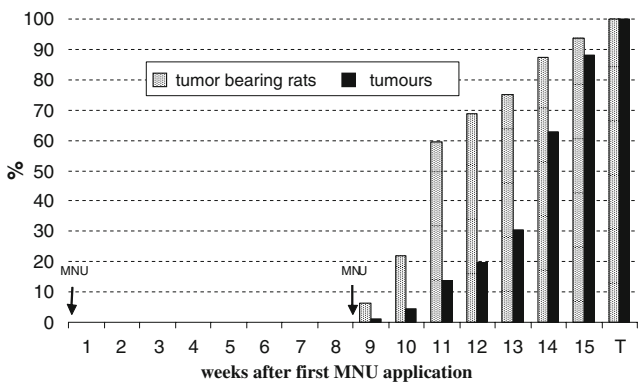

Fig. 1 The proportion of tumour bearing animals and proportion of total tumours

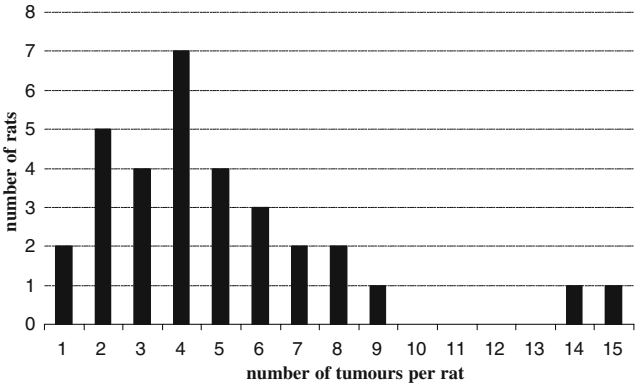

Fig. 2 Frequency of mammary tumours in MNU-induced rats

Tumour Histology

Mammary tumours were identified as adenomas (6/158) and carcinomas (152/158). The majority of carcinomas were invasive (137/152; 87%) and only a minority were *in situ* ductal carcinomas (15/152). Of the invasive carcinomas, 4% mammary tumours ulcerated the skin, 14% contained large areas of necrosis and haemorrhage and 34% invaded the local neighbouring tissues such as muscle, salivary gland or lymph nodes. Metastases were observed in a distant lymph node in one rat and in the liver and spleen in the other (Fig. 4). Of the 32 MNU-induced rats, 29 developed invasive carcinomas, two developed carcinomas *in situ* and one, lactating adenomas.

Various combinations of papillary, cystic, cribriform or solid patterns of ductal carcinomas occurred in tumours. Tubular carcinomas appeared rarely (9/137). The most typical and frequent of the MNU induced carcinomas were mixed papillary-cribriform pattern. Invasive carcinomas were characterised by penetration into the surrounding stroma of finger-like projections or duct-like structures or solid sheets of epithelial cells and by broken basement membrane. Massive stromal response, demonstrated by inflammatory infiltration and fibrosis, was frequently observed. A large area of necrosis was often observed in invasive carcinomas.

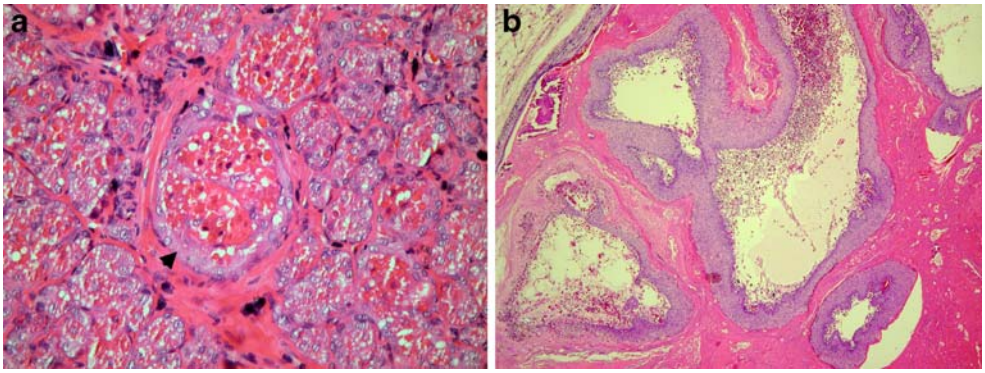

Fig. 3 Clitoral gland of rat. **a** Normal clitoral gland. The ductuli are covered by squamous epithelium (arrow). **b** Squamous cell papilloma of a clitoral gland after MNU application. Papillary structures are covered by differentiated squamous epithelium of ducts

Ulcerated Tumours

Ulcerated tumours first developed 14 weeks after the first MNU application. However, each rat with an ulcerated tumour developed only one ulcerated mammary tumour, although tumour multiplicity of those rats ranged between 2 and 6 tumours per rat. The weight of mammary tumours, which ulcerated the skin (4% of all invasive carcinomas) ranged from 8.83 g to 16.9 g per tumour. It appears that the expanse of tumour is not the most important factor in aggressiveness (ulceration of the skin) as the number of large, non-ulcerated mammary tumours (ranging between 9.63 g and 14.46 g) was similar. Histological examination revealed that in ulcerated tumours mostly solid-cribriform

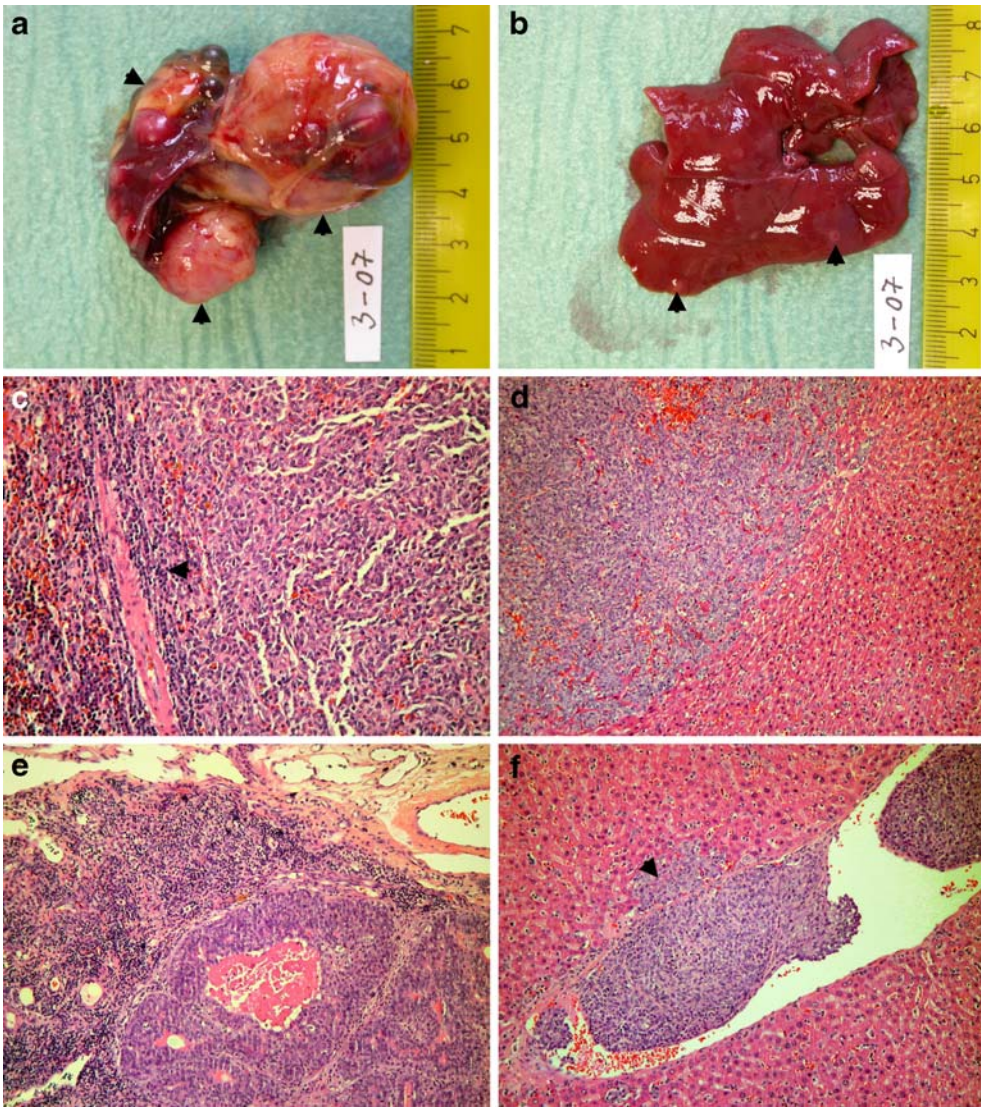

Fig. 4 Metastases of MNU-induced mammary carcinomas to distant organs. **a** Gross photograph of spleen with multiple metastases of different size (*arrows*). **b** Gross photograph of liver with disseminated small subcapsular metastases (*arrows*). **c** Metastasis to the spleen. There is an insignificant margin between the tumour tissue and the spleen parenchyma (*arrow*). **d** Metastasis to the liver. There is an irregular margin between the tumour and liver tissue; the latter is compressed by tumour tissue. **e** Metastasis to the lymph node. The carcinoma tissue is well demarcated from adjacent lymphatic tissue. **f** Blastomic thrombus in the hepatic vein with an invasion of the hepatic parenchyma (*arrow*)

patterns were observed, in contrast to the mostly mixed cribriform-papillary patterns in large, non-ulcerated tumours. A solid pattern was also observed in invasive carcinomas that invaded the surrounding tissue.

Discussion

There is substantial evidence suggesting that the MNU animal model mimics human breast cancer in terms of the rat tumour's histopathology, origination from mammary ductal epithelial cells, dependence on ovarian hormones for tumour development, and altered expression of TGFB, erbB2, cyclin D1 [11]. The MNU induced breast cancer model has therefore been used extensively to evaluate preventive and therapeutic agents for human breast cancer [12–15]. Chemically induced rodent models are also suitable for studying malignant progression [2]. To investigate the advanced stages of mammary gland tumours, Liska [7] established and compared two protocols of MNU administration depending on the animal's age and the number of applications of carcinogen.

We chose one of Liska's [7] breast cancer models, but terminated the experiment 2 weeks earlier than proposed [7]. The MNU-induced rats developed large necrotizing tumours that ulcerated the skin (6/32). All rats in the experiment were euthanized within a week, 14 weeks after the first MNU application, and thorough examination was performed.

In contrast to Liska [7], who observed 76% incidence of palpable carcinomas after 16 weeks, 87% incidence of palpable carcinomas was observed in our case, 14 weeks after the first MNU application. Moreover, in our experiment further histological examination of mammary gland revealed that all rats developed mammary neoplasms, of which all except one (31/32) developed carcinomas. One rat developed lactating adenomas. Interestingly, in our experiment tumour incidence (5 tumours per rat) was considerably higher than that reported by Liska (2 tumours) [7], who observed a relatively high variability in tumour incidence between rats. However, in our case, variation in tumour multiplicity was even higher. Nevertheless, in our experiment the histological type of mammary carcinomas was mostly mixed papillary-cribriform, in agreement with other findings [6, 7].

We observed that all palpable lesions were malignant and most of them were locally aggressive, invading surrounding tissue. Earlier macroscopic appearance and faster growth is one of the characteristics of the malignant character of tumours, as observed by many researchers [4, 6]. In our experiment, histological examination revealed that 87% of all carcinomas found were invasive, which is nearly twice as many as reported [7]. Moreover, in our experiment some of the MNU-induced mammary tumours with faster growth rate ulcerated the skin in 14 weeks after the first MNU application, which was not reported by Liska et al. [7]. Nevertheless, it has been shown that ulceration of mammary tumours is characteristic of MNU-induced tumours and is affected by the dose of carcinogen [6]. However, MNU induced mammary tumours are not only locally aggressive but are also able to metastasize to distant organs. Several investigators observed metastases to spleen, liver, lungs [4, 8, 16] and bone marrow [17]. Although Liska [7] did not report metastasis in distant organs, we observed metastasis to spleen, liver and lymph node in 6% of rats (2/32), 14 weeks after the first MNU application.

Recently, Chan and co-workers [11] showed that rat mammary carcinomas appear closely related to human invasive carcinomas in general, and in particular, to lower grade, ER-positive human tumours. They proposed that the MNU induced rat model could be used to study the transition of this subset of breast cancer from an *in situ* lesion to invasive cancer [11]. Our results, showing induction of a large number of invasive carcinomas in a relatively short time, indicate that this protocol offers induction of an appropriate MNU breast cancer rat model for studying malignant progression of mammary tumours.

It is well known that each animal model has its specific advantages and disadvantages. The main drawback of the breast cancer rat model considered here is the considerable variation in tumour incidence in the rat that can occur between laboratories, or even between experiments in the same laboratory, regardless of the route or dose of carcinogen [3]. One of the main reasons for variation could be the seasonal influence caused by variation in pineal melatonin production and immune function in rodents under otherwise constant environmental conditions [18–20]. Loscher and co-workers [18] studied mammary carcinogenesis in female Sprague–Dawley rats at different seasons of the year but under constant environmental conditions (photoperiod, temperature, air humidity, food). They observed (the experiment was performed twice within 2 years) that the experiment performed in autumn yielded a significantly lower tumour incidence and tumour burden than the same one performed during spring/summer time [18]. Moreover, it was recognised that not only time of the year but also time of the day could influence mammary carcinogenesis. It has been shown that tumour incidence is higher when carcinogen is administered in the late afternoon than in the morning [21]. It is suggested that, besides strain, diet, age, dose, time of the day and year of the carcinogen administration, and immune and endocrine system status, there are other still unknown factors that may cause changes in induction of tumorigenesis in rodents under identical conditions [22].

In addition, Lu and co-workers [23] have shown that multiple carcinomas induced by MNU and detected by palpation within a given animal arise from multiple, independently initiated cells, which emerge as distinct mammary carcinomas in that animal. Therefore, variation in tumour incidence may be influenced, not only by environmental factors, but also by genetic factors of the individual animal. Since outbred Sprague Dawley rats were mostly used as model of MNU induced breast cancer, genetic variations between rats of that strain could be one of the important reasons of variability in tumour incidence.

It has been reported that tumours of the clitoral gland occur spontaneously, mainly in aged animals (15–30 months) [24], but can also be induced by carcinogens [25]. However, to our knowledge, tumours of the clitoral gland have not been observed under MNU induction alone. However, it was observed that rats treated with MNU and mammosomatotropic pituitary tumour developed a high incidence of clitoral gland hyperplasia [26], but not neoplasm as was observed in one solitary rat treated with MNU alone in our experiment.

Regarding animal welfare, which is an important component of animal experiments, the weight or expanse of individual or total tumour mass per rat is important data. The expanse of mammary tumours can influence animal behaviour or even the animal's health. The influence on an animal's welfare can be even greater in the case of malignant necrotizing tumours, which often ulcerate. Considering the weight of single tumour or total tumour burden per rat (which, in our experiment, reached over 10% of the animal's weight) and the invasive character of half of the malignant carcinomas, animal welfare could be a matter of debate [27]. We have shown that MNU treated rats had significantly increased numbers of neutrophils, showing an inflammatory response of rats with large mammary tumours. Therefore, we propose that use of this model should be restricted to studying malignant progression. It is a matter, not only of animal welfare or human endpoints, but also of the validity of scientific results that can be confounded because of the unsuitable state of an animal's health or of factors other than those investigated.

Conclusions

The MNU induced rat model is widely used in breast cancer research, mostly for evaluating preventive and therapeutic agents for human breast cancer. Although substantial evidence suggests that it mimics human breast cancer in many respects, the MNU model also has disadvantages. It is a non-negligible factor to know that tumour incidence and tumour burden could be influenced by circa-annual rhythms, irrespective of environmental conditions. Furthermore, considerable variation in tumour incidence, regardless of the route or dose of carcinogen administration, environmental conditions, or even time of the year, is one of its main drawbacks. Therefore, it is recommended that only the number of tumours per rat or per group, and the number or incidence of malignant tumours is useful in drawing conclusions from obtained results. Further, our results lead us to caution against use of the MNU model other than for studying malignant progression.

References

1. Parkin DM, Bray F, Ferlay J et al (2005) Global cancer statistics, 2002. CA Cancer J Clin 55:74–108
2. Clarke R (1996) Animal models of breast cancer: their diversity and role in biomedical research. Breast Cancer Res Treat 39:1–6
3. Russo J, Russo IH (1996) Experimentally induced mammary tumors in rats. Breast Cancer Res Treat 39:7–20
4. McCormick DL, Adamowski CB, Fiks A et al (1981) Lifetime dose-response relationships for mammary tumor induction by a single administration of *N*-methyl-*N*-nitrosourea. Cancer Res 41:1690–1694
5. Isaacs JT (1986) Genetic control of resistance to chemically induced mammary adenocarcinogenesis in the rat. Cancer Res 46:3958–3963
6. Thompson HJ, Adlakha H (1991) Dose-responsive induction of mammary gland carcinomas by the intraperitoneal injection of 1-methyl-1-nitrosourea. Cancer Res 51:3411–3415
7. Liska J, Galbavy S, Macejova D et al (2000) Histopathology of mammary tumours in female rats treated with 1-methyl-1-nitrosourea. Endocr Regul 34:91–96
8. Thompson HJ, Adlakha H, Singh M (1992) Effect of carcinogen dose and age at administration on induction of mammary carcinogenesis by 1-methyl-1-nitrosourea. Carcinogenesis 13:1535–1539
9. Thompson HJ, McGinley JN, Rothhammer K et al (1995) Rapid induction of mammary intraductal proliferations, ductal carcinoma in situ and carcinomas by the injection of sexually immature female rats with 1-methyl-1-nitrosourea. Carcinogenesis 16:2407–2411
10. Russo J, Russo IH (2000) Atlas and histologic classification of tumors of the rat mammary gland. Journal of Mammary Gland Biology and Neoplasia 5:187–200
11. Chan MM, Lu X, Merchant FM et al (2005) Gene expression profiling of NMU-induced rat mammary tumors: cross species comparison with human breast cancer. Carcinogenesis 26:1343–1353
12. Badawi AF, Eldeen MB, Liu Y et al (2004) Inhibition of rat mammary gland carcinogenesis by simultaneous targeting of cyclooxygenase-2 and peroxisome proliferator-activated receptor gamma. Cancer Res 64:1181–1189
13. Kubatka P, Ahlers I, Ahlersova E et al (2003) Chemoprevention of mammary carcinogenesis in female rats by rofecoxib. Cancer Lett 202:131–136
14. Mehta RG (2000) Experimental basis for the prevention of breast cancer. Eur J Cancer 36:1275–1282
15. Roomi MW, Roomi NW, Ivanov V et al (2005) Modulation of *N*-methyl-*N*-nitrosourea induced mammary tumors in Sprague–Dawley rats by combination of lysine, proline, arginine, ascorbic acid and green tea extract. Breast Cancer Res 7:R291–R295
16. Rivera ES, Andrade N, Martin G et al (1994) Induction of mammary tumors in rat by intraperitoneal injection of NMU: histopathology and estral cycle influence. Cancer Lett 86:223–228

17. Gullino PM, Pettigrew HM, Grantham FH (1975) *N*-nitrosomethylurea as mammary gland carcinogen in rats. J Natl Cancer Inst 54:401–414
18. Loscher W, Mevissen M, Haussler B (1997) Seasonal influence on 7, 12-dimethylbenz[a]anthracene-induced mammary carcinogenesis in Sprague–Dawley rats under controlled laboratory conditions. Pharmacol Toxicol 81:265–270
19. Sumova A, Bendova Z, Sladek M et al (2004) Seasonal molecular timekeeping within the rat circadian clock. Physiol Res 53 Suppl 1:S167–S176
20. De Jonage-Canonico MB, Lenoir V, Martin A et al (2003) Long term inhibition by estradiol or progesterone of melatonin secretion after administration of a mammary carcinogen, the dimethyl benz (a)anthracene, in Sprague–Dawley female rat; inhibitory effect of Melatonin on mammary carcinogenesis. Breast Cancer Res Treat 79:365–377
21. Halberg F (1964) Grundlagenforschung zur Ätiologie des Karzinoms. Ärztl Fortb 2:67–77
22. Kubatka P, Ahlersova E, Ahlers I et al (2002) Variability of mammary carcinogenesis induction in female Sprague–Dawley and Wistar:Han rats: the effect of season and age. Physiol Res 51:633–640
23. Lu J, Jiang C, Mitrenga T et al (1998) Pathogenic characterization of 1-methyl-1-nitrosourea-induced mammary carcinomas in the rat. Carcinogenesis 19:223–227
24. Reznik G, Ward JM (1981) Morphology of neoplastic lesions in the clitoral and prepucial gland of the F334 rat. J Cancer Res Clin Oncol 101:249–263
25. Eustis SL, Haseman JK, Mackenzie WF et al (1995) Toxicity and carcinogenicity of 2, 3-dibromo-1-propanol in F344/N rats and B6C3F1 mice. Fundam Appl Toxicol 26:41–50
26. Ito O, Okamoto T, Fujimoto N et al (1994) Inhibition of mammary tumours by pretreatment with 17 beta-estradiol in F344 rats induced with *N*-methyl-*N*-nitrosourea. Jpn J Cancer Res 85:279–283
27. Workman P, Balmain A, Hickman JA et al (1988) UKCCCR guidelines for the welfare of animals in experimental neoplasia. Lab Anim 22:195–201