

## RESEARCH ARTICLE

# Correlation of Tumour Response with Starting Tumour Size and Dose of Tamoxifen in an N-Methyl-N-Nitrosourea (NMU)-Induced Rat Mammary Cancer Model

Hassan Muhammad Yankuzo<sup>1</sup>, Sharifah Tuan Sheriff Emilia<sup>2</sup>, Rumaizi Shaari<sup>3</sup>, Nik Soriani Yaacob<sup>1\*</sup>

### Abstract

**Background:** The aim of this preliminary study was to address variations of responses observed with different starting tumor sizes of 10 and 15 mm, and the effects of different doses of tamoxifen (TAM) on experimental rat mammary tumors. **Materials and Methods:** Thirty-five inbred female Sprague Dawley rats aged 43 days were administered with three weekly doses of N-methyl-N-nitrosourea (NMU) intraperitoneally (ip) at 50 mg/kg body weight. Animals were randomized (beginning from 10 mm tumor size) into four TAM-treated (50, 100, 200 and 500 µg/day) groups of six animals each, and another group (n=6) treated with TAM 100 µg/day at starting tumour size of 15 mm. The animals were treated by oral gavage daily for 8 weeks before sacrifice. **Results:** Serum urea and creatinine, and overall physical tumor burden were significantly modulated in animals treated with variable doses of TAM compared to the untreated controls (n=5). Final body weight and tumor number were significantly different in the 10 mm-treated animals compared to those treated at 15 mm. There were no significant differences in histopathological features among all the groups. **Conclusions:** Our findings suggest the importance of standardizing tumour size and drug doses before initiation of treatment, particularly in the direct comparison of basic end-tumour physical parameters.

**Keywords:** NMU - rat mammary tumor - tamoxifen - tumor size

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### Introduction

Breast cancer is the commonest cancer among women in the United States and the second leading cause of cancer deaths in the women worldwide (after lung cancer) with estimated 232,340 new cases of invasive breast cancer and 39,620 deaths among US women in 2013 (De Santis et al., 2013). However, the global trend of breast cancer burden varies between regions and countries. According to the report of Porter (Porter, 2009), the trend of breast cancer burden was particularly more evident in the low- and middle-income countries where 45% incidence rate and 55% breast cancer-related deaths were estimated from over 1 million cases diagnosed worldwide in 2009.

In Malaysia, breast cancer is also the commonest cancer constituting 18.1% of the reported cancer cases, and the leading cause of cancer related mortality among women within 40-49 years (Yip et al., 2006). It has been estimated that 1 out of every 20 women in Malaysia had 1 in 20 chance of developing breast cancer in her lifetime, and the incidence was highest in the Chinese, followed by Indians and Malays (Rampal and Yahaya, 2008; Dahlui et

al., 2011; Moore, 2013).

Experimental animal models (particularly rodents) are commonly used in the study of breast cancer because of their biological similarity with human breast cancer in terms of epithelial origin, hormonal (estrogen) dependence and gene expression profiles (Chan et al., 2005). Chemically-induced breast cancer rat model is notably a simple and standard laboratory model for the study of human breast cancer (Tsubura et al., 2011). For such model, the chemical carcinogens N-Methyl-N-Nitrosourea (NMU) and 7,12-dimethyl-benzanthracene (DMBA), are most frequently used for breast cancer induction. NMU is particularly known to induce estrogen-dependent mammary tumours in female Sprague Dawley rats (Soares-Maia et al., 2013).

Tamoxifen (TAM) has been the gold standard anti-estrogen drug of choice for prevention and treatment of hormone dependent breast cancer in the high risk women (Jordan, 2012). But TAM is also known to have many side effects (Karimi et al., 2009) that limit its routine clinical application. Studies on the effects of TAM on experimental rat mammary tumours have been reported using variable

<sup>1</sup>Departments of Chemical Pathology and <sup>2</sup>Pathology, School of Medical Sciences, Universiti Sains Malaysia, Kubang Kerian,  
<sup>3</sup>Department of Surgery, Faculty of Veterinary Medicine, Universiti Malaysia Kelantan, Kelantan, Malaysia \*For correspondence: [niksoriani@usm.my](mailto:niksoriani@usm.my)

doses of the antiestrogen. Most of these studies have focused on the chemoprevention effects (Manni et al., 2010) or complications of long term adjuvant treatment, including development of TAM resistance (Motamedi et al., 2012) and tendencies to induce carcinogenic effects (Behtash et al., 2009).

Tumour size is an established independent predictor in the management of breast cancer patients (Orang et al., 2013; Chin et al., 2014). However unlike in the clinical setting, it is interesting that convenient tumour size for initiating treatment on experimental animal models can easily be standardized. Yet variable methodologies, particularly the starting tumour size and dose of TAM have been reported in many literature reports; which lead to some degree of inconsistencies on the end-tumour response data. It was reported that treatments were mostly initiated at starting tumour sizes between 5 mm to 15 mm (Goss et al., 2007; Nishino et al., 2009; Ahlem et al., 2011).

To our knowledge to date, no experimental rat mammary tumor-regression study has been conducted specifically to correlate the effect of variations in starting tumor size with the end-tumor response data. Thus this study was initiated to help clarify the variations of TAM dose and tumor size in NMU-induced rat mammary tumor model at the start of treatment. The study aimed to highlight the importance of standardizing starting tumor size and drug dose before initiation of treatment with potential anticancer agents. This may help to achieve uniform end-tumor response data and also improve the natural course of treatment response.

## Materials and Methods

### Animals

Inbred female Sprague Dawley rats (*Rattus norvegicus*) (body weight 100 g to 150 g) were obtained from the Animal Research and Service Centre (ARASC), Universiti Sains Malaysia (USM). The animals were housed (in pairs) in the polycarbonate cages with 2 inches thick of pinewood chip bedding for absorption of their excreta. The rats were acclimatized to standard vivarium conditions (temperature 23±2°C, relative humidity 70±5%, and 14 h light-10 h dark cycle) for 1 week. They were fed with rodent pellet (Gold Coin Feedmills Sdn. Bhd. Malaysia) and had free access to tap water *ad libitum*. Environmental enrichment using tissue paper rolls and rubber toys were occasionally provided to the animals. The study was performed following the standards and guidelines as approved by the USM Animal Ethics Committee with reference number USM/AEC/2011/(69)(304).

### Induction of breast cancer

Breast cancer was induced by NMU (Sigma, MO, USA), injected intraperitoneally in three weekly doses of 50 mg/kg body weight starting from 43 days of age. NMU was freshly dissolved in a warm physiologic saline (25 mg/ml), adjusted to pH 4.0 using 3 % acetic acid and maintained at 35–40°C just before administration. The age-matched control rats received 0.9% physiologic saline. All rats were weighed weekly following carcinogen treatment, and their mammary glands were palpated twice weekly for detection of tumours starting from 3 weeks after the first

NMU injection. Palpable tumours appeared within 6-9 weeks post induction and tumour growth was measured using a digital vernier caliper. Tumour number, size and volume were also recorded weekly.

### Experimental design

Two experimental protocols were utilized. The first protocol was to compare the effect of variable doses (50, 100, 200, and 500 µg) of TAM. For this purpose animals were randomised (at 10 mm tumour size) into four treatment groups (n=6 per group) and an untreated control group (n=5). Tamoxifen (free base, Sigma, MO, USA) solution was prepared by first dissolving in absolute ethanol (10 mg/ml) followed by suspension in the corn oil. The ethanol was evaporated under low stream nitrogen gas before use. Daily doses were aliquoted in small eppendorf tubes covered with aluminum foil and kept at -20°C. TAM was administered by oral gavage daily for eight weeks, beginning at the initial tumour size of 10 mm. Response to treatment was assessed weekly by measurement of the animal body weight and tumour growth variables.

Next protocol was to compare the end tumour responses in animals that were initiated treatment with TAM 100µg/day for 8 weeks at the starting tumour size of 10 mm and 15 mm. To achieve this, a group of animals (n=6) were treated with TAM 100 µg daily for 8 weeks at the starting tumor size of 15 mm and compared with the ones that begin treatment at 10 mm tumour size.

Existing tumours at the start of treatment were closely monitored throughout the study period, and the appearance and growth of new tumours were recorded and followed up. Untreated animals were sacrificed 8 weeks after detection of a 10 mm tumour or earlier if the tumour size reached 40 mm. All rats were monitored for signs of physical distress (e.g., reduced food and water intake, labored breathing, restricted movements and persistent recumbence posture). Other major complications like infected ulcer on the tumour surface, high tumour burden impairing normal movement of the animal, bleeding orifices and/or significant weight loss (more than 20% weight lost after induction) were also monitored. In the event of any complications, appropriate interventions under supervision of veterinary specialists were offered where necessary.

### Tumor volume measurement

To analyze changes in tumour growth following treatment with TAM, the tumour volume was monitored weekly by measuring tumour length (a) and width (b) (in millimeter) at right angles using a digital Vernier caliper. The tumour volume (mm<sup>3</sup>) was estimated using Carlson's formula  $V = ab^2/2$ , where "a" and "b" represent the longest and shortest tumour diameters respectively. The tumour volume for each rat was quantified as total volume of all sizeable nodules in order to represent the actual tumour burden.

### Animals sacrifice and samples preparation

Animals were euthanized after 8 weeks of treatment using i.p. phenobarbitone (Alfasan, BV, Holland) at a dose of 50 mg/kg. Blood was immediately collected by

cardiac puncture into plane vacutainer tubes and allowed to coagulate for about 1 hour at room temperature. The blood was centrifuged at 10,000Xg for 10 minutes at 4°C to separate the serum, plasma, and red blood cells. Serum analyses of liver enzymes (Alanine and aspartate transaminases), renal function profile (electrolytes, urea, creatinine, uric acid), and calcium levels were conducted using automated chemistry analyzer (Architect, c8000) to ascertain side effects from our interventions, if any.

In order to define and compare histopathological changes in the mammary tumours after 8 weeks of treatment, representative sections of each group were subjected to routine haematoxylin and eosin (H & E) staining. Briefly, the excised tumours were fixed in 10% neutral buffered formalin for 24 hours and processed overnight for paraffin embedding. Sections of 3 µm were cut, rehydrated in xylene and graded ethanol solutions and stained with H & E. The specimens were examined by a pathologist and changes were graded according to Nottingham modification of the Bloom and Richardson method, where extent of tubule formation, nuclear pleomorphism and abnormal mitotic figures were evaluated (Meyer et al., 2005).

#### Statistical analysis

Data analysis was conducted using SPSS Version 20 (IBM Corp) and the results for tumour growth variables and animal body weight were expressed as median and interquartile range (IQR). Multiple Man Whitney test with Bonferroni correction was used to compare differences in the end tumour burden between untreated tumour bearing animals (controls) and animals treated with variable doses of TAM. Statistical differences between starting treatment at 10 mm and 15 mm starting tumour size were compared by using Man Whitney test. Comparisons with  $p < 0.05$  were considered to be statistically significant.

## Results

#### Effect of variable doses of TAM on the end tumour burden

Animals treated with 100, 200 and 500 µg TAM show significant dose-dependent reduction in the final number

of tumor nodules and the end tumor volume compared to the untreated controls (Table 1). The values of mean tumor weight at necropsy ( $1.25 \pm 0.35$ ,  $0.31 \pm 5.89$ ,  $0.00 \pm 2.17$ ) were also reduced (although not statistically significant) compared to the untreated group ( $2.14 \pm 9.12$ ). In contrast the animals treated with TAM 50 µg/day did not show significant effect on any of the tumour growth parameters measured compared to untreated controls. Generally all animals had marginal increase in body weight throughout the experiment and comparison of mean change in body weights show non-uniform disparity between TAM-treated and untreated groups.

Nonetheless, two rats each from the group treated with TAM 50 µg/day and 200 µg/day, developed infected ulcer at about 7 weeks of treatment and subsequently lost significant body weight. These animals were thus humanely euthanized. The remaining NMU-induced rats without tumour formation after 25 weeks post induction were excluded from the analysis

#### Effect of variable doses of TAM on biochemical parameters

Serum renal profile, levels of liver enzymes and calcium were determined to assess any possible side effects resulting from the intervention. However, no significant changes were observed in all the parameters above between TAM-treated animals and the untreated controls (data not shown).

#### Histopathological changes in NMU-induced rat mammary tumors

The H & E staining of the representative mammary tumour sections (Figure 1) indicated invasive adenocarcinomas in both untreated control group and the tumor sections treated with variable doses of TAM. However, the phenotype in the treated groups were of slightly less aggressive subtypes (cribriform carcinoma) compared to that of untreated control. Cribriform carcinoma showed clusters of epithelial cells with intense desmoplasia and lymphocytic infiltrations at the periphery. In addition, most of the sections displayed numerous cystic changes typical of human adenoid cystic carcinoma (ACC), a rare type of breast cancer comprising of about

**Table 1. Effect of Variable doses of TAM on the end Tumor Burden Median (IQR)**

Group	Final tumour number	Change in tumour volume (mm <sup>3</sup> )	Tumour weight (g)	Change of body weight (g)
Untreated	2.0 (3.0)	1,225.0 (24,349.0)	2.1 (9.1)	25.0 (82.0)
TAM 50	2.0 (2.0)	770.0 (3,189.0)	2.1 (9.4)	-1.5 (22.0)
TAM 100	1.0 (1.0)*	-46.0 (127.0) *	1.3 (0.4)	28.0 (12.0)
TAM 200	0.0 (1.0)*	-173.0 (489.0)*	0.3 (5.9)	-1.0 (21.0)
TAM 500	0.0 (1.0)*	-149.0 (123.0)*	0.0 (2.2)	3.0 (21.0)

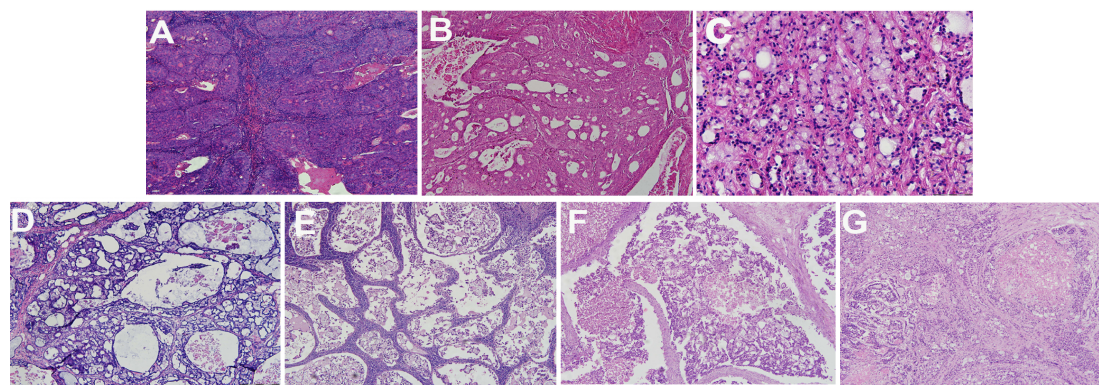
Data were analyzed by Multiple Man Whitney with Bonferroni correction ( $\alpha/4$  comparisons); \*Significantly different from untreated control ( $p < 0.05$ ); TAM 50: Tamoxifen 50 µg/day; TAM 100: Tamoxifen 100 µg/day; TAM 200: Tamoxifen 200 µg/day; TAM 500: Tamoxifen 500 µg/day; Negative score implies significant shrinkage of the tumour at the end of treatment compared to the initial tumour volume

**Table 2. Comparison of end Tumor Response according to the Starting Tumor Size**

Group	Final tumour number	Change in tumour volume (mm <sup>3</sup> )	Tumour weight (g)	Change of body weight (g)
Untreated	2.0 (3.0)	1,225.0 (24,349.0)	2.1 (9.12)	25.0 (82.0)
10 mm	1.0 (1.0)*	-46.0 (127.0)	1.3 (0.4)	28.0 (12.0)*
15 mm	2.0 (2.0)	1,371.0 (8,072.0)	4.4 (6.5)	2.5 (12.0)

Data were analyzed by Man-Whitney test; \*Significantly different from 15 mm-treated animals ( $p < 0.05$ ); 10 mm: Animals initiated on TAM 100 µg/day at starting tumor size of 10 mm; 15 mm: Animals initiated on TAM 100 µg/day at starting tumor size of 15 mm





**Figure 1. NMU-Induced Rat Mammary Tumor Sections Stained with Hematoxylin & Eosin.** No significant histomorphological variations between untreated tumors and animals (bearing NMU-induced mammary tumors) treated with variable doses of TAM at starting tumor size of 10 or 15 mm. Mammary tumors were harvested after 8 weeks of treatment with variable doses of TAM, and representative sections from each group were stained with hematoxylin & Eosin (H & E) for comparison purposes as described in the materials and methods section. (a-b) Sections of tumor untreated at 10 mm and 15 mm tumor sizes, n=5 rats. (c) Tumor section of the rats treated with TAM 50  $\mu$ g/day showing grade 2 invasive carcinoma with prominent cystic changes. (d-e) Tumor sections of the rats treated with TAM 100  $\mu$ g/day at starting tumor sizes of 10 mm and 15 mm, features were consistent with grade 2 invasive carcinoma, with additional moderate foci of central necrosis (comedo type of necrosis). (f-g) Sections of relapsed tumors from the rats treated with TAM 200 and 500  $\mu$ g/day showing many foci of central necrosis and other features consistent with grade 2 invasive carcinoma. All sections were captured at 10 x magnification, and scored by modified Scarff-Bloom-Richardson (SBR) scoring index. (c-g) n=6 rats per group

0.1% human breast cancer (Tavassoli and Devilee, 2003). The phenotype of the untreated control sections displayed epithelial clusters of solid cells round in shape, with moderate increase in the nucleo-cytoplasmic ratio, moderate stromal desmoplasia, moderate foci of necrosis and some poorly differentiated structures. Predominantly the histopathological features observed in the untreated tumours were consistent with grade 3 while the tumours treated with variable doses of TAM were consistent with grade 2 based on modified Scarff-Bloom-Richardson (SBR) scoring of invasive adenocarcinoma.

#### *Impact of starting tumor size on the treatment response*

In order to determine the impact of tumour size on treatment response, animals treated with TAM 100 $\mu$ g/day at the starting tumour size of 10 mm were compared with those that received the same dose at a larger starting tumour size of 15 mm. The final number of tumour nodules were significantly reduced in the group of animals that were initiated on treatment at 10 mm tumour size compared to the group of 15 mm starting tumour size (Table 2). A marked reduction in the end tumour volume was also observed in the 10 mm group, although it was not statistically significant due to the large IQR values.

No significant differences were observed when serum liver function and renal function profiles of animals treated with TAM at 10 mm and 15 mm size were compared, except for the incidental findings of high serum alkaline phosphatase level and significantly higher urea level (data not shown) in the 15 mm tumour group which may reflect initiation of liver and renal injuries (Usunomena et al., 2012) and therefore high tendencies of unfavorable outcome with late initiation of treatment. Likewise comparison of histomorphology of the TAM-treated 10 and 15 mm tumor sections was found to be essentially similar (grade 2) except for occasional comedo necrosis and reactive lymph nodes that were more prominent in

the 15 mm compared to 10 mm tumours.

## **Discussion**

Chemical-induced rat mammary tumours are commonly employed in the study of human breast cancer. The biological behavior of chemical-induced rat mammary tumours has been extensively reported (Al-Daheri et al., 2008). NMU-induced animal model is frequently used in view of its close similarities with human breast cancer in terms of ductal epithelial cells origin, malignant features, hormonal contents and significant response to ovariectomy or anti-estrogen therapy (Cardiff, 2001). Previous *in vivo* reports using this animal model have shown dose-dependent antitumour effect of TAM as a chemopreventive agent when administered over a long period of time (Jordan, 1983; Manni et al., 2010).

In the current study, NMU-induced mammary tumours were first developed in the animals and the tumour growth response to TAM treatment was investigated. Our results indicate that reduction in the number of tumour nodules and the end tumour volume in response to TAM treatment is dose-dependent with significant effects observed with a minimum TAM dose of  $\geq 100\mu$ g/day. This is in line with a previous report on the antitumour activity of TAM 100  $\mu$ g/kg in a similar animal model following eight weeks of treatment at the starting tumour size of  $>10$  mm (non-specified) (Goss et al., 2007). In a study analyzing the chemopreventive effect of variable doses of TAM, Gottardis and Jordan (1987) described TAM 100 $\mu$ g/day as an effective anti-tumour dose. In that study, partial response was demonstrated with TAM 25 $\mu$ g/day following eight weeks treatment and complete tumour-free state was achieved with TAM 100 $\mu$ g/day after 23 weeks of continuous treatment. On the other hand, our study showed no significant changes in the tumour growth parameters following treatment with TAM 50 $\mu$ g/day

compared to the untreated group for the same treatment period. However this is in contrast to a similar report on the chemoprevention study where TAM 50µg/day was reported to maintain the animals in a complete tumour-free state after 170 days of continuous treatment (Jordan, 1983). This probably implies that while a low TAM dose can be effective as a chemopreventive agent, a higher TAM dose is required to cause regression of established mammary tumours. Alternatively, a longer treatment duration with lower TAM doses such as 50µg/day could be as effective in causing tumour regression and this represents a limitation of the current study. The phenotypic tumour changes (cribriform carcinoma) observed in the treated groups in our study is also in line with a report on the mechanism of apoptotic cell death induced in a similar animal model following 5 days treatment with Rapamycin, platelet factor 4 and their combination (Idris et al., 2014).

There was also non-uniform disparity between animals treated with variable doses of TAM and untreated group throughout the experiment. In contrast, TAM was reported to have suppressive effect on the body weight due to its selective oestrogen-like property on energy balance, thereby reducing food consumption, body fat contents and skeletal system (Fudge et al., 2009). In addition, Rodrigues and colleagues (2012) reported significant decrease in body and uterine weights of adult female rats after 30 days of daily treatment with TAM 250 µg/day. Therefore lack of significant body weight loss in our data may be attributed to low dose of TAM administered over a short treatment period.

Previous studies involving chemoprevention or combination treatment designs have reported variable starting tumor sizes such as 5 mm (Ahlem et al., 2011), 6 mm (Melancon et al., 2005), greater than 10 mm (not specified) (Goss et al., 2007) and 15 mm (Nishino et al., 2009), at initiation of treatment with TAM or other antiestrogens. The two parallel studies of Haag and Gould (1994) and Haag et al., (1992) have described palpable tumour nodules of 4-5 mm and those of ≥ 10 mm as early and advanced mammary tumours, respectively. In addition, Jaafar and colleagues (2009) investigated the relationship between tumour size and phenotype of NMU-induced rat mammary tumours and reported that tumours of ≤ 8.0±0.5 mm were predominantly benign lesions, while those of tumour size ≥ 12.0±0.5 mm were malignant. Thus the choice of 10 mm and 15 mm starting tumor sizes in the current study is ascribed to the consideration of advanced tumor stages at these tumor sizes comparable to that of advanced human breast cancer (Cardiff, 2001).

Overall, our results indicate favorable prognosis when NMU-induced rats were initiated on treatment at the starting tumour size of 10 mm compared to 15 mm. The mean number of tumour nodules at the end of TAM treatment duration was significantly less in the 10 mm tumour group. This is also reflected in the mean tumour volume although the difference was not found to be statistically significant due to the large intra group variability. In addition, elevation of total serum protein, urea and alkaline phosphatase in the animals initiated on treatment at 15 mm compared to those initiated at 10 mm (although statistically non-significant), may reflect a mild

non-specific liver function impairment (Usunomena et al., 2012) that was however suppressed due to short treatment duration or other variable host factors.

Considering the known positive response of hormone-dependent mammary tumors to antiestrogens and the pilot study nature of this report, some of the limitations therefore include lack of assessing the hormonal status of non-responding tumours and perhaps the short treatment duration. It is possible that the non-responding mammary tumours were estrogen-receptor negative (ER-ve) irrespective of starting tumour size, or perhaps better response could have been obtained from ER+ve tumours if the treatment was prolonged. On the other hand, the effect of TAM ≥ 100µg could only be suppressing the tumour growth (tumouristatic) rather than effectively killing the tumour cells (cytotoxic), and the latter may be reactivated by normal hormonal response afterwards. In view of these limitations, our observations need further validation and must be interpreted with caution.

Nevertheless, responses to the combination of a drug and a potential anticancer agent may vary between different doses of the drug used and high concentrations of the drug may also mask the activity of the potential anticancer agent. The size of the tumour at the initiation of treatment could influence the treatment outcome and variable sizes of the starting tumour have been adopted in various studies, making comparisons difficult. The current study has therefore highlighted the importance of standardizing the drug dose and the tumour size for analysis of potential anticancer agents especially when determining possible synergistic effects of multiple combinations.

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