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ORIGINAL ARTICLE

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The effect of keratinocyte growth factor on tumour growth and small intestinal mucositis after chemotherapy in the rat with breast cancer

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Abstract *Purpose:* Mucositis from cancer chemotherapy is a common problem for which there is no definitive treatment. It produces significant morbidity and occasional mortality. Prevention and successful treatment could significantly enhance the quality of life of patients, and improve survival; however any potential preventative agent must not enhance tumour growth. The aims of this study were to assess the effect of keratinocyte growth factor (KGF) on breast tumour growth, and in preventing small intestinal mucositis induced by methotrexate (MTX). *Methods:* Tumour-bearing rats received KGF or saline for 5 days prior to either MTX or saline treatment, and were killed 24 h after the last MTX injection. The weights of the tumour, small and large intestines, and liver were recorded. Apoptosis was assessed by TUNEL assay in the tumour and jejunum. Intestinal morphometry was used to assess villus area, crypt length and mitotic crypt count. Tumour proliferation was assessed by mitotic count. *Results:* KGF increased the weight of the small intestine prior to chemotherapy but the weight was not maintained after chemotherapy. KGF synergized with MTX to increase apoptosis in both intestinal crypts and the breast cancer. KGF also reduced tumour size. *Conclusions:* We conclude that KGF had a modest effect on intestinal growth prior to chemotherapy. It did not protect the gut from mucositis, nor did it worsen morphometry. It reduced tumour size.

Keywords Keratinocyte growth factor · Chemotherapy · Mucositis · Breast cancer · Rats

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Introduction

Mucositis is a clinical term that is used to describe the side effects of cancer chemotherapy affecting mucosal surfaces such as that of the small intestine. Typical symptoms include bloating, abdominal pain and diarrhoea. Approximately 40% of all cancer patients receiving chemotherapy develop mucositis, and 60% of patients receiving high-dose chemotherapy develop mucositis (one half of these patients will need modification of their treatment or will require parenteral analgesia) [11, 15]. Whilst major progress has been made in the understanding and treatment of toxicity to bone marrow from chemotherapy, little progress has been made in preventing mucositis. There is no effective treatment of established mucositis other than relieving symptoms using good oral hygiene and analgesics. Mucositis limits the dose of cancer chemotherapy that can be given [5, 10, 11, 12].

Studies in laboratory animals have shown that cytotoxic drugs and radiation cause apoptosis and hypoproliferation in intestinal crypts [5, 12, 13, 14, 17, 18]. All cytotoxic drugs and radiation treatment cause apoptosis [7] which is not always dose dependent [19]. Our studies in human cancer patients have demonstrated that mucositis is caused by apoptosis and hypoproliferation of intestinal crypts following chemotherapy [12]. Apoptosis of intestinal crypts peaks with a ninefold increase on day 1, whereas hypoproliferation reaches a nadir on day 3 after chemotherapy when compared to pretreatment values. Intestinal damage is evident as mild

villous atrophy, crypt hypoplasia, reduced mitosis and abnormal intestinal permeability that peaks from 3 to 7 days after chemotherapy [10, 11, 12].

Keratinocyte growth factor (KGF) belongs to the heparin-binding growth factor family [1, 2, 9, 20] and differs from other family members in having a specific trophic action on epithelial cells [1]. It is secreted by mesenchymal cells in a paracrine manner [1, 9]. KGF receptor is present throughout the gastrointestinal tract [4, 9, 16]. Treatment of rats with doses of 1 to 5 mg/kg KGF causes growth of the liver, stomach and small and large intestines [9]. Farrell and colleagues have shown that KGF protects mice from combined chemotherapy and radiation-induced gastrointestinal injury and improves mortality when administered prior to cytotoxic chemotherapy [4, 5]; however there was no benefit of KGF treatment after chemotherapy. The aims of this study were to investigate the effect of KGF in protecting against intestinal mucositis due to chemotherapy, and on tumour growth, using the laboratory rat implanted with breast cancer. Our specific hypothesis was that treatment with KGF should reduce apoptosis, or ameliorate hypoproliferation, in the gastrointestinal tract without compromising the effect of chemotherapy on the cancer.

Methods

Preparation of breast cancer cell suspension

Mammary breast cancer was donated by Dr. A. Rofe (Institute of Medical and Veterinary Sciences, Adelaide, South Australia). The breast cancer used in this study was a spontaneous, rat-specific, mammary adenocarcinoma that arose in the 1970s. A tumour-bearing model mimics the human situation more closely, and allows assessment of potential tumour protection. Three female donor rats were injected subcutaneously into both flanks with 0.2 ml (2.0×10^7 cells per ml) tumour inoculum 11 days prior to the beginning of the study, with these cells subsequently forming a tumour. The animals were killed by CO_2 asphyxiation and cervical dislocation. The subcutaneous tumours were dissected from the rats and placed in sterile PBS. The animal work described was approved by the Animal Ethics Committee at The Women's and Children's Hospital, Adelaide. The work and animal care complied with the National Heath and Medical Research Council Code of Practice of Care for Animals in Research and Teaching (1997).

Preparation of tumour inoculum

Diced pieces of tumour were homogenized and filtered through sterile gauze. The tumour cell suspension was spun at 1100 *g* four times for 3 min each time, with the PBS being decanted off and replaced between each spin. Viable cells were counted using 0.5% trypan blue.

Experimental plan

The study was divided into two parts. First, the effect of KGF on intestinal growth in rats with breast cancer was investigated. This was to verify the dose, duration and biological effect of KGF in this model. Second, the effect of KGF on ameliorating the small intestinal mucositis caused by methotrexate (MTX) was investigated.

Verification of effect of KGF on intestinal growth

A group of 28 female DA rats weighing approximately 150 g were implanted with a tumour inoculum of 4.0×10^6 cells in 0.2 ml into each flank and the tumours were allowed to grow for 9 days. The rats were treated with daily subcutaneous injections of 3 mg/kg KGF for either 3 or 5 days before being killed. The weights of the small and large intestines and of the breast cancer were recorded (Table 1). Jejunal samples were taken for morphometric analysis.

Effect of KGF in preventing intestinal mucositis

A group of 38 female DA rats weighing approximately 150 g were anaesthetized with 4% isoflurane and injected subcutaneously with 4.0×10^6 cells in 0.2 ml into each flank. After 48 h, rats were individually caged and randomly assigned to six equal groups. Three

Table 1. Effect of KGF treatment for 3 or 5 days on the weight of the small and large intestines, liver and tumour. The values shown are mean weights (SE) in grams in groups of four rats

Group	Small intestine	Large intestine	Liver	Tumour
Saline 3 days	4.564 (0.11)	1.422 (0.05)	6.451 (0.09)	1.145 (0.13)
Saline 5 days	4.439 (0.18)	1.193 (0.08)	6.785 (0.65)	3.077 (1.02)
KGF 3 days	6.030 (0.24)**	1.583 (0.10)	11.617 (0.62)*	0.858 (0.11)
KGF 5 days	6.678 (0.27)*	1.436 (0.07)	10.074 (0.22)**	2.362 (0.55)

*$P<0.05$, **$P<0.025$, vs respective saline treatment

Table 2. Weight of small and large intestines, liver and breast cancer in female DA rats. Rats were treated for 5 days with KGF or left untreated (control). Rats were treated with MTX and killed 24 h after the last dose. Day 0 is the first day of chemotherapy administration and day 2 is 24 h after chemotherapy has been completed. Animals killed on day 0 received no chemotherapy. The values shown are mean weights (SE) in grams in groups of six rats

Group	Small intestine	Large intestine	Liver	Tumour
Day 0/KGF	5.745 (2.35)*	1.370 (0.56)	11.241 (4.59)	4.016 (1.64)
Day 0/Control	4.262 (1.74)	1.353 (0.55)	6.549 (2.67)	6.386 (2.61)
Day 2/KGF/MTX	4.307 (1.63)**	1.423 (0.54)[a]	7.923 (2.99)	2.986 (1.13)***
Day 2/–/MTX	3.285 (0.15)	1.224 (0.55)	6.929 (3.10)	5.122 (2.29)
Day 2/KGF/–	5.015 (2.05)[a]	1.355 (0.55)	8.764 (3.58)	10.098 (4.12)
Day 2/Control/–	4.025 (1.52)	1.295 (0.49)	7.543 (0.29)	10.769 (4.07)

*P <0.05, **$P<0.034$, ***$P<0.0013$, vs respective treatment without KGF
[a]Not significantly different from respective treatment without KGF

groups were treated daily for 5 days with subcutaneous KGF (3 mg/kg) and three groups with saline (Table 2).

Two control groups (one receiving KGF; one saline) were killed after 5 days to determine the biological effect of KGF on the gut. The gastrointestinal tract from the pyloric sphincter to the rectum was dissected, and the wet weights of the small and large intestines, and liver were measured. Samples of small intestine of length 2 cm at 25% of the length of the small intestine from the pylorus were collected and placed into Clark's fixative and in 10% formalin for further analysis. The tumours were removed and weighed. Cross sections of non-necrotic tumour were dissected and placed in 10% formalin. The remaining four groups of animals received either intramuscular 1.5 mg/kg MTX or saline injections daily for 2 days before being killed 24 h after the second injection. This MTX regimen had previously been validated in this model [12].

Intestinal morphometry

Small sections of jejunum were opened onto cardboard, fixed in Clark's fixative for 24 h and stored in 70% ethanol. The stored tissue was rehydrated and hydrolysed in 1 *M* HCI for 7 min at 60°C. After two washes in double-distilled water, the tissue was stained with Schiff's reagent for 45 min before being microdissected using a stereomicroscope. The tissue was examined following mounting in 45% acetic acid, and, using a calibrated graticule, measurements were made of the villus length, the apical and basal widths of 15 villi and the lengths of 15 crypts. Villus area was calculated using a trapezoid approximation as previously described [3].

TUNEL assay

Sections of tumour and small intestine were fixed for 24 h in 10% formalin and stored in 70% ethanol. Tissue was dehydrated by passing through a graded series of ethanol and embedded in paraffin wax. Sections (4 μm) were cut using a microtome and placed in an oven at 60°C overnight. The method for labelling the apoptotic cells was that of Gavrieli et al. [6]. For the tumour, apoptotic cells were counted and are expressed as the number of apoptotic bodies observed per square millimetre of tissue. In the jejunum, apoptotic bodies were expressed as a percentage of apoptotic cells per crypt.

Proliferative assessment of tumour

Cross-sections of tumour were fixed in 10% formalin and embedded in paraffin. Histological sections were cut (5 μm) and the number of mitotic cells per square unit area were counted after staining with haematoxylin and eosin. Counts were taken from the periphery to avoid the necrotic centre of the tumour. Tumour proliferation refers to the number of mitotic cells observed per square millimetre of tissue.

Statistical analysis

The Peritz' *F*-test, which is a robust measure of differences between group means where the number in each group is not necessarily equal [8], was used for all statistical analyses.

Results

Verification of effect of KGF on intestinal growth

KGF was biologically active, and increased the wet weights of the small intestine by 32% and 50% after 3 and 5 days of treatment, respectively (Table 1). Similarly, the wet weights of the large intestine and liver were also increased. KGF increased villus area by 20–40%, and crypt proliferation by 25–35% (Fig. 1). Unexpectedly, it caused an approximately 25% reduction in tumour weight (Table 1). Our results indicated that KGF was biologically active because it increased organ weight and small bowel proliferation. The biological effect of KGF was greater after 5 days than after 3 days of pretreatment.

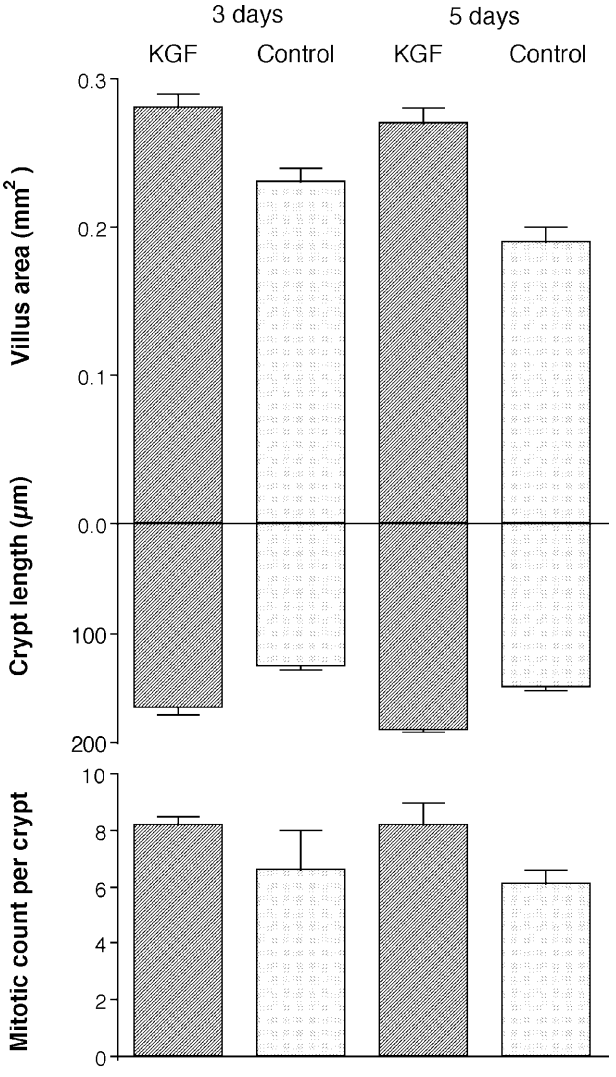

Fig. 1. Effect of KGF treatment for 3 and 5 days on intestinal morphometry in rats with breast cancer

Effect of KGF on preventing intestinal mucositis

KGF protected the small intestine from MTX-induced reduction in wet weight (4.3 g vs 3.3 g, $P < 0.034$, Table 2). We did not detect any significant difference in villus area between control and KGF-treated groups at 2 days, but KGF increased crypt length ($P < 0.034$) and mitotic count per crypt ($P < 0.05$). However, there were no significant differences in intestinal morphometry between animals treated with KGF prior to MTX treatment and those treated with MTX (group 3 vs 4, Fig. 2).

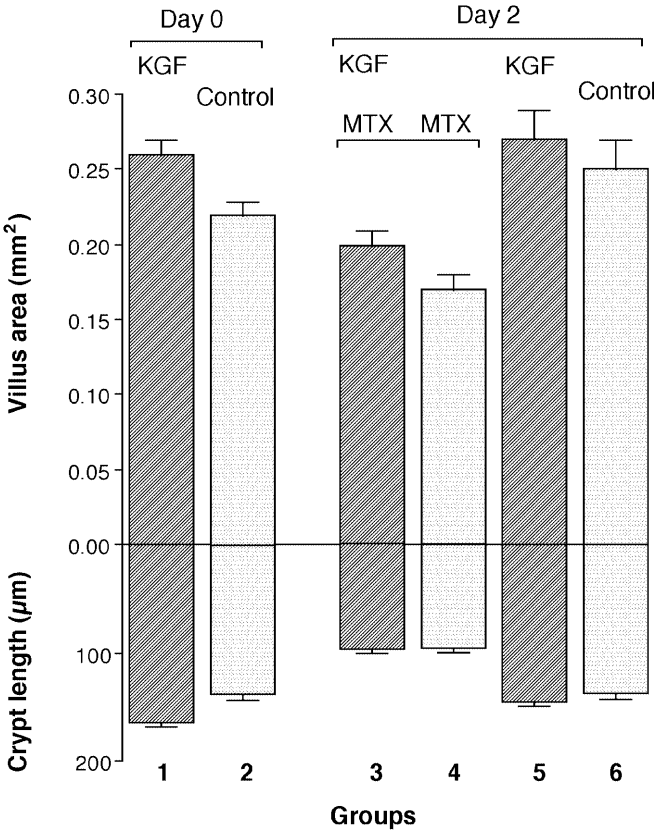

Fig. 2. Effect of KGF treatment for 5 days followed by MTX on intestinal morphometry in rats with breast cancer. The final dose of KGF was given on day-1

KGF treatment alone had no effect on jejunal apoptosis (group 1 vs 2, Fig. 3), but synergized with MTX to increase jejunal apoptosis (group 3 vs 4, Fig. 3) as well as apoptosis in the breast cancer (group 3 vs 4, Fig. 4).

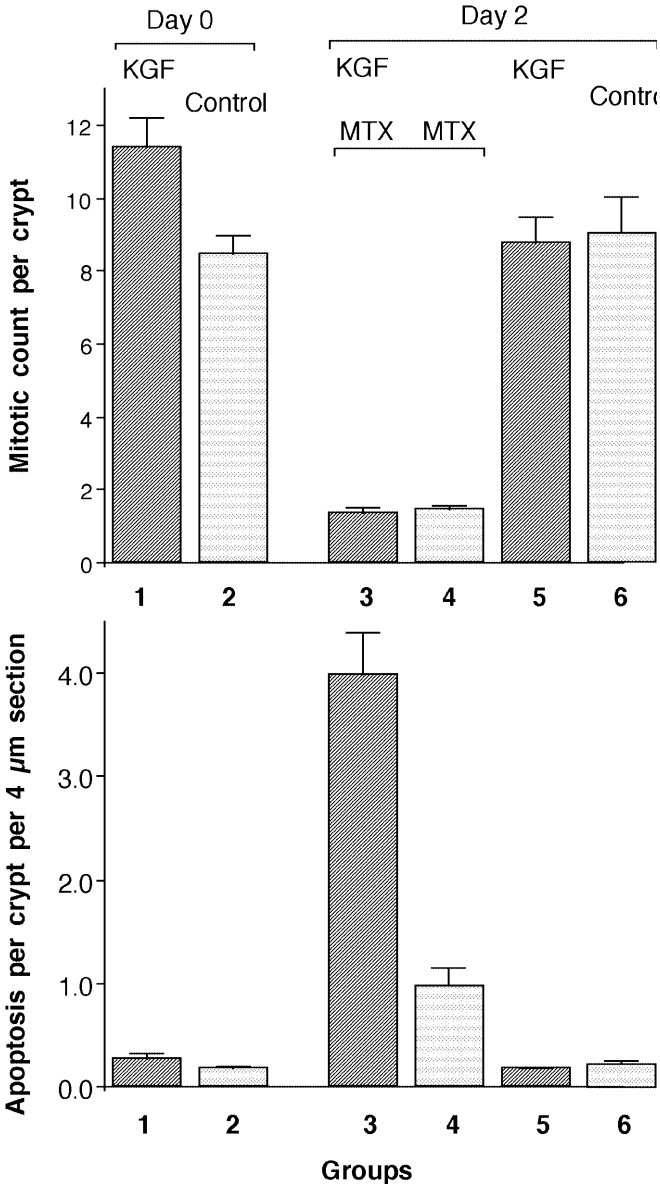

Fig. 3. Effect of KGF treatment for 5 days followed by MTX on intestinal crypt proliferation (mitotic count per crypt) and on apoptosis of intestinal crypts

Discussion

Prevention of mucositis by KGF pretreatment was investigated in this study rather than accelerating repair because it is better to prevent symptoms and morbidity. Mucositis was assessed by apoptosis in intestinal crypts and by intestinal morphometry. Our findings showed that KGF primed the small intestine before MTX treatment and maintained small intestinal weight but did not lead to an improvement in intestinal morphometry compared to MTX treatment alone. KGF and MTX also increased apoptosis in intestinal crypts and in breast cancer. KGF did not promote tumour growth.

Many epithelial tumours express the KGF receptor and might therefore grow in response to KGF [19, 20]. However, KGF did not promote growth of the breast cancer used in this study. The breast cancer used in this study originally arose spontaneously in DA rats and has been subsequently passaged. It is not known whether it expresses KGF receptors. Our experimental model of subcutaneously implanted breast cancer allowed us to easily measure tumour burden, proliferation and apoptosis. The majority of previous experimental studies of mucositis have not considered the confounding effect of cancer, although in one experiment in a study by Farrell et al. implanted human colon cancer was used in nude mice [4]. It is essential that any treatment for mucositis does not promote tumour growth.

The effectiveness of KGF in protecting the small intestine from chemotherapy-induced mucositis was investigated. KGF pretreatment prior to MTX treatment resulted in maintenance of intestinal weight, but did not protect against chemotherapy-induced mucositis, as there was no difference in villus area, crypt length or mitotic indices (Fig. 3). Thus KGF treatment 'primed' gut growth prior to MTX treatment, and led to

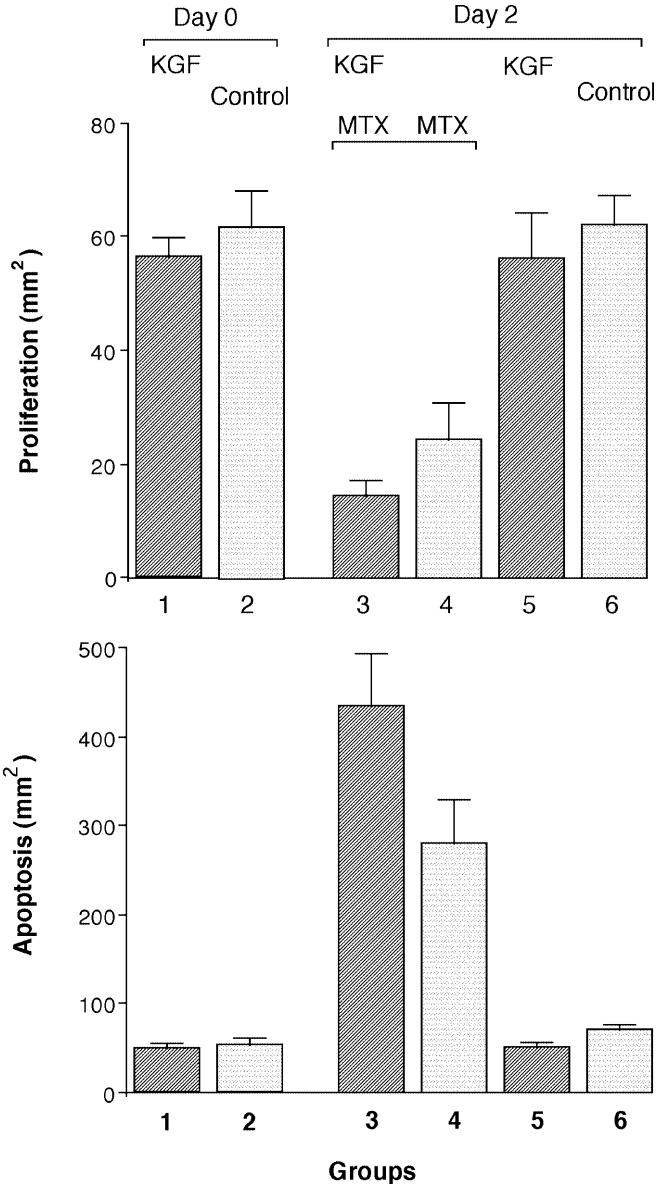

Fig. 4. Effect of KGF treatment for 5 days followed by MTX on tumour proliferation and on apoptosis

preservation of intestinal wet weight following MTX treatment, but did not improve intestinal morphometry or apoptotic rate following MTX treatment. The results of our study differ from those of Farrell et al. [4] who showed that KGF treatment led to maintenance of villus and crypt lengths equivalent to those of control animals when given to mice prior to 5-flourouracil. The explanation for the different findings of the two studies is unclear. Rats implanted with breast cancer were always used in our study, whereas nude mice implanted with human cancer were only used in some of the experiments in the study by Farrell et al. Neither the differences between rats and mice, nor our use of MTX vs 5-fluourouracil, would seem to explain the difference.

Apoptosis increased in the jejunum and in the tumour after MTX treatment. Animals receiving KGF prior to MTX treatment had a further fourfold increase in apoptosis in the jejunum, but this did not worsen intestinal damage at least on day 2. We plan to investigate whether this increase in apoptosis leads to a worsening of intestinal damage at a later time.

Rather than KGF enhancing tumour growth, it synergized with MTX to reduce tumour burden presumably by increasing tumour apoptosis (Fig. 4). In animals that received KGF, tumours were approximately 40% lighter than in animals receiving only MTX. Farrell et al. [4] also considered the possibility that KGF could promote tumour growth but found no evidence for this in their in vivo mouse model. Our study extended this work by showing that KGF reduced tumour burden, but at the expense of increased apoptosis in the small intestine.

We conclude that while KGF does not protect the small intestine from morphological changes following chemotherapy, it does lead to preservation of the wet weight of the small intestine following chemotherapy, as well as synergizing with MTX to reduce tumour burden in the rat with breast cancer.

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