

## RESEARCH ARTICLE

# Green Tea Polyphenol Protection Against 4-Nitroquinoline 1-Oxide-Induced Bone Marrow Lipid Peroxidation and Genotoxicity in Wistar Rats

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### Abstract

4-Nitroquinoline 1-oxide (4-NQO) a potent oral carcinogen, widely used for induction of oral carcinogenesis, has been found to induce lipid peroxidation *in vivo* and *in vitro*. Green tea contains a high content of polyphenols, which are potent antioxidants. Thus green tea polyphenols (GTP) might be expected play a protective role against 4-NQO induced lipid peroxidation and bone marrow toxicity. In the present study, a dose of 200 mg of GTP/kg b.wt/day was given orally for a week, simultaneously animals received 0.2 ml of 0.5% 4-NQO in propylene glycol (5 mg/ml) injected intramuscularly for three times/week. Oxidants and antioxidants such as malendialdehyde (MDA) and thiols, glutathione peroxidase (GPx), glutathione reductase (GR), superoxide dismutase (SOD) and catalase (CAT) were significantly decreased in 4-NQO induced animals except MDA, and these parameters were brought back to near normalcy on treatment with GTP. The results suggest that GTP treatment offers significant protection against 4-NQO induced lipid peroxidation and bone marrow toxicity and might be a promising potential candidate for prevention of mutations leading to cancer.

**Keywords:** Green tea polyphenols - 4-nitroquinoline 1-oxide - bone marrow - lipid peroxidation

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### Introduction

4-NQO is a water soluble oral carcinogen which produces papilloma and invasive squamous cell carcinoma, resulting in clinical and histologic changes which is similar to those observed in neoplasms of humans (Fisker, 1990). 4-NQO is known to induce multi-step carcinogenesis. 4-NQO is also known to induce H-ras mutation in chromosome 7 leading to head and neck squamous cell carcinoma in experimental murine model, which is quite similar to that of tumours that develop in tobacco chewers (Hendler et al., 1996; Srinivasan et al., 2004; Srinivasan et al., 2006; Srinivasan et al., 2007).

In experimental animals, the oral mucosa is relatively resistant to the carcinogenic effects of test compounds (Ito, 1981). However, application of 4-nitroquinoline-N-oxide (NQO) directly to the rat tongue (painting) or in the drinking water (Tanaka et al., 1993) induces squamous cell carcinoma. The potent activity of NQO provides a useful model for oral carcinogenesis (Eveson, 1981). Subcutaneous administration of NQO yielded lung adenoma, uterine leiomyosarcoma, soft tissue sarcoma and fibro sarcoma, etc., administration of NQO by stomach tube, yielded adenocarcinoma, papilloma and

squamous cell carcinoma. NQO is a pluripotent carcinogen and its organ specificity depends strongly on route of administration. NQO is a strong mutagen in standard short-term assays and induces DNA single-strand breaks in mammalian cells (Walker & Sridhar, 1976).

4-NQO is also cytotoxic at sub-micro molar concentrations, as observed in clonogenic assays with mammalian cell lines (Goth-Goldstein et al., 1984; Tsuda, 1990). Nitroreductases (DT diaphorase/NADPH-quinone oxidoreductase and other mammalian enzymes (Benson, 1993) activate NQO by reduction to 4-hydroxylaminoquinoline-1-oxide (HAQO). The distribution of reductase activity within the oral cavity, in rats, correlates with the distribution of squamous cell carcinomas induced by NQO (Booth, 1990). O-Acetylation of HAQO generates a reactive acetoxy ester which forms three major covalent DNA adducts: N<sub>2</sub>-guanine, C<sub>8</sub>-guanine, and N<sub>6</sub>-adenine (Bailleul et al., 1989).

4-NQO can be detoxified by reaction with glutathione (GSH), either spontaneously or catalyzed by GSH transferases (GST; primarily  $\mu$  and Pi classes) to give 4-(glutathion-S-yl) quinoline 1-oxide (Aceto et al., 1990). GST enzymes protected human MCF-7 cells from the

genotoxic and mutagenic effects of NQO, although they failed to protect these cells from its cytotoxicity (Fields et al., 1994). Free radical-mediated genotoxicity may also contribute to the action of NQO on cells. HAQO cleaves DNA *in vitro* in the presence of Cu (II) (Yamamoto et al., 1993), and catalase, but neither OH• radical scavengers nor superoxide dismutase, inhibited this cleavage. The active species causing DNA damage may be formed from the reaction of hydrogen peroxide and Cu(I). Univalent reduction of NQO causes superoxide production, via redox-cycling catalyzed by the enzymes cytochrome P<sub>450</sub> reductase and xanthine oxidase, but not DT-diaphorase (Fann et al., 1999). NQO is a powerful inducer of the SoxRS oxidative stress response in *E. coli* (Nunoshiba et al., 1993) and studies in the yeast *Saccharomyces cerevisiae* also indicate that reactive oxygen species contribute to NQO toxicity (Ramotar et al., 1998).

Green tea is a popular beverage consumed in some parts of the world and is a rich source of polyphenols, which are antioxidant in nature (Katiyar & Mukhtar, 1996). Green tea contains many polyphenols known as catechins, including epigallocatechin-3-gallate (EGCG), epigallocatechin (EGC) and epicatechin-3-gallate (ECG) (Jankun et al., 1997). Epidemiological studies have associated the consumption of green tea with a lower risk of several types of cancer including stomach, oral cavity, esophagus and lung. In fact, tea is one of the few agents that can inhibit carcinogenesis at the initiation, promotion and progression stages (Katiyar & Mukhtar, 1996). Green tea polyphenols has antioxidant / free radical quenching activity, with EGCG being the most potent. Earlier reports postulate that gallyl groups may provide themselves to increasing antioxidant effectiveness. Green tea polyphenols is a potent antioxidant compound that has demonstrated greater antioxidant protection than vitamin C, E, rosemary extract and curcumin in experimental studies (Katiyar & Mukhtar, 1996). Catechins, especially EGCG, can ameliorate free radical damage to DNA, under certain conditions (Anderson et al., 2000).

Despite there has been growing body of evidence postulating the role of Green tea polyphenols against 4-NQO in oral (Srinivasan et al., 2004; Srinivasan et al., 2006; Srinivasan et al., 2007). Hence, the present investigation was designed to study the effect of GTP on 4-NQO induced free radical alteration on bone marrow and its genotoxic nature.

## Materials and Methods

4-NQO was purchased from Sigma Chemical Company (St. Louis, MO, USA). Fresh Green tea leaves were collected from The Nilgris, Tamil Nadu, India. All other chemicals used were of analytical grade.

### Preparation of GTP

Extraction of green tea polyphenols (GTP 80%) was achieved by adapting the procedure of Shaowen Lee (Director, Human King long Bioresource Co. Ltd., China) followed by Srinivasan et al. (2006, 2007). GTP was characterized by UV, IR and HPLC (Srinivasan et al., 2007).

### Animals

Wistar male albino rats (10 weeks old) weighing 80 to 120 gm were purchased from TANUVAS (Chennai, India). The animals were housed, four per cage in a room with controlled temperature and humidity with 12 h light: dark cycles. All the animals were given a standard rat feed (Hindustan Lever Ltd., Bangalore) and water *ad libitum*. This study was conducted as per the guidelines of the animal ethical committee.

### Experimental protocol

The animals were divided into 4 groups, each group comprising of 6 animals of similar weight. The animals were provided with autoclaved pellet food and water and scorings of the animal activity were recorded.

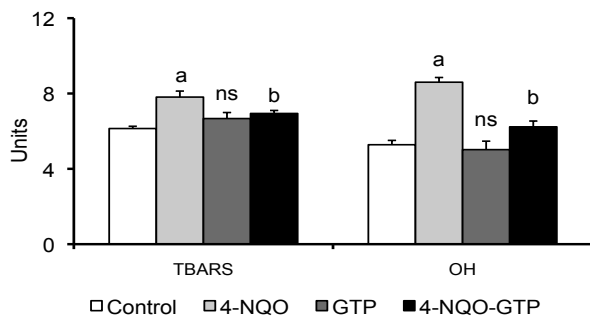
**Group-I** – Control animals. **Group-II** – (Induced) The animals received 0.2 ml of 0.5% 4-NQO in propylene glycol (5mg/ml) injected intramuscularly in inner thigh region for three times/week. **Group-III** – (Drug control) The animals received 200 mg of GTP/kg b.wt/day orally for a week. **Group-IV** – (Treated) The animals received 4-NQO same as group II and GTP as Group III. The experiment was terminated at the end of the week.

After the experimental period, the animals were anesthetized using ether, sacrificed by cervical decapitation. The thigh femur bone was removed and the bone marrow was aspirated using fine needle and used for the present study. A portion of bone marrow was subjected to histological studies.

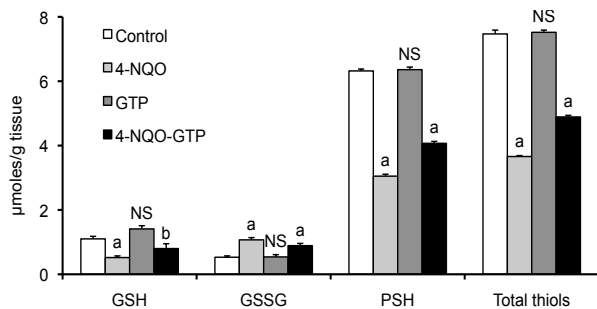
The level of lipid peroxides was estimated by the method of Ohkawa et al. (1979). The level of lipid peroxides is expressed as n moles of TBARS/mg protein. Hydroxy radical production was measured by the method of Cederbaum and Cohen (1984). The amount of formaldehyde was expressed as ng/mg of protein. Superoxide dismutase was assayed following the method of Misra and Fridovich (1972) and expressed as 50% inhibition of epinephrine auto oxidation/min/mg protein in tissue. Catalase was assayed according to the method of Takahara et al. (1960) and expressed as  $\mu$ moles of H<sub>2</sub>O<sub>2</sub> decomposed/ min/mg protein. Glutathione reductase was assayed by the method of Staal et al. (1969) and expressed as nmoles of NADPH oxidized /min/mg protein under incubation conditions. The activity of glutathione peroxidase was assayed by the method of Rotruck et al. (1973). Glutathione peroxidase activity is expressed as n moles of glutathione oxidised/min/mg protein. Glutathione S-transferase was assayed by the method of Habig et al. (1974). The enzyme activity was expressed as nmoles of CDNB conjugated/min/ mg protein.

GSH was estimated by the method of Moron et al. (1979). GSSG was measured by the method of Griffith (1980). Total thiol content was estimated by the method of Sedlack and Lindsay (1968). Protein thiols were calculated by subtracting the non-protein thiols from total thiols.

Immunohistochemistry was carried out as conventional method, but the difference in immunoperoxidase staining is by Zhang et al. (2002) method, using anti-MDA monoclonal antiserum (D10A1). MDA-DNA adducts were counted using a Nikon Y-FL ECLIPSE 400 (Japan) microscope connected to a Nikon FDX-35 camera (Japan).



**Figure 1. Effect of GTP on the Levels of Oxidants in Control and Experimental Groups.** Values are expressed as mean  $\pm$  SD. Comparisons: Control Vs 4-NQO, 4-NQO Vs 4-NQO + GTP, a -  $p < 0.001$ , b -  $p < 0.01$ , ns - Non significant. Units-LPO;  $\mu$ mol of MDA/mg of protein, OH; ng/mg of protein.



**Figure 2. Effect of GTP on the Levels of Cellular Thiols in the Bone Marrow of Control and Experimental Groups.** Values are expressed as mean  $\pm$  S.D (n=6). On comparing 4-NQO, GTP with control - a  $p < 0.001$ , NS - non significant. On comparing 4-NQO with 4-NQO+GTP- a  $p < 0.001$ , b  $p < 0.01$ .

To measure the relative intensity scoring was done as arbitrary units 4 as intensely stained, 3 as moderately stained, 2 as mild staining, 1 as poorly stained in control and experimental groups.

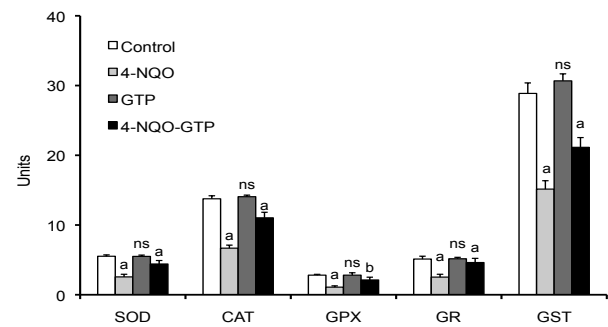
#### Statistical analysis

Statistical analysis was done using SPSS (Statistical Package for Social Science) (10.0) computer software. Differences with calculated  $p < 0.001$ ,  $p < 0.01$ ,  $p < 0.05$  were regarded as significant.

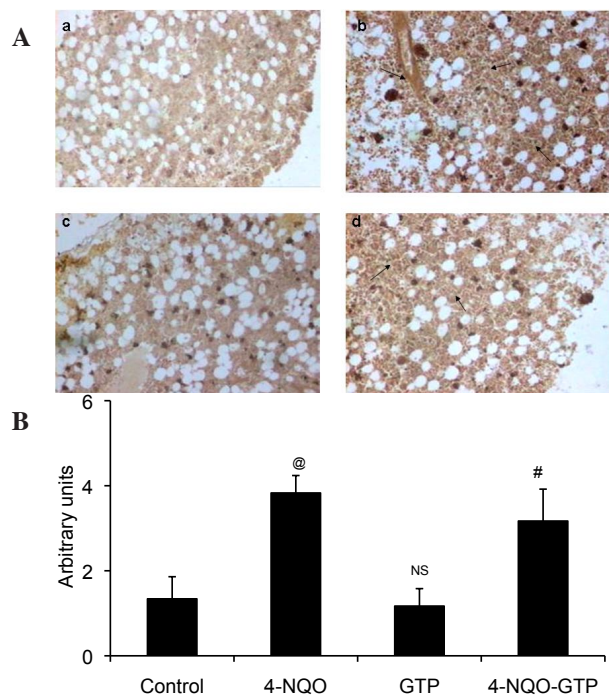
## Results

Figure 1 shows the effect of GTP on the levels of oxidants in control and experimental groups. The levels of TBARS and hydroxyl radicals were significantly ( $p < 0.001$ ) increased in 4-NQO-induced animals. A significant ( $p < 0.001$ ) reduction in the levels of TBARS and hydroxyl radicals was observed in 4-NQO + GTP animals. In 4-NQO treated group showed a significant depletion of GSH, PSH and total thiols (Figure 2) were as a significant ( $p < 0.001$ ) increase in GSSG was observed when compared to control animals. Simultaneous supplementation of GTP to 4-NQO treated group (group 4) tends to decrease the oxidative stress produced by 4-NQO, which moderately maintains the level of GSH, PSH and total thiols which was found to be statistically significant ( $p < 0.001$ ).

The enzymic antioxidant profile of GTP treated to



**Figure 3. Effect of GTP on the Status of Enzymic-Antioxidant in Bone Marrow of Control and Experimental Groups.** Values are expressed as mean  $\pm$  SD (n=6). On comparing 4-NQO, GTP with control - a  $p < 0.001$ , NS - non significant. On comparing 4-NQO with 4-NQO+GTP- a  $p < 0.001$ , b  $p < 0.01$ . SOD-50% autooxidation of epinephrine/min, CAT- $\mu$ g of  $H_2O_2$  consumed/min, GPX- $\mu$ g of glutathione consumed/min, GR- $\mu$ g of NADP+ formed/min



**Figure 4. Effect of GTP on MDA-DNA adduct formation in control and experimental groups of animals.** (A): a: (Control) Normal expression of MDA-DNA adduct, b: (4-NQO) high expression of expression of MDA-DNA adduct was observed, c: (GTP) Expression was similar to that of control, d: (4-NQO+GTP) lesser degree of expression of MDA-DNA adduct was noticed. -> shows the expression of MDA-DNA adduct. (B): Values are expressed as mean  $\pm$  S.D (n=6). On comparing 4-NQO, GTP with control - @  $p < 0.05$ , NS - non significant. On comparing 4-NQO with 4-NQO-GTP- #  $p < 0.05$ .

4-NQO-induced toxicity in bone marrow was shown in Figure 3. The levels of SOD, CAT, GPx, GR and GST were found to be significantly ( $p < 0.001$ ) decreased in 4-NQO induced rats when compared with control animals. The altered enzyme activities were comprehensively brought back to the near normalcy in the case of SOD, GPx, GST and GR. A non-significant change was observed in all parameters in GTP alone treated group of animals when compared to that of control animals, this proves the non-

toxic nature of GTP.

MDA-DNA adducts in the control and 4-NQO treated animals were shown in Figure 4. In 4-NQO induced group, there was a significant ( $p < 0.001$ ) increase in MDA-DNA adduct formation as compared to control group. In case of GTP treated to 4-NQO treated animals significant ( $p < 0.001$ ) decrease in MDA-DNA adducts was noted as compared to 4-NQO treated animals. The quantification of MDA-DNA adduct positive cells were shown in Figure 4B.

## Discussion

4-NQO is also known to cause intracellular oxidative stress (Ramotar et al., 1998), which leads to lipid peroxidation. 4-NQO is known to cause tissue damage in three hours of administration is known to cause acute toxicity, mutagenicity or carcinogenicity by inducing a pro-oxidant state in vivo which leads to lipid peroxidation, ultimately producing OH and MDA. MDA is the major reactive aldehyde resulting from the peroxidation of biological membrane (Vaca et al., 1998). MDA is a secondary product of LPO, used as an indicator of tissue damage by a series of chain reaction (Ohkawa et al., 1979).

Oxidative stress is defined as "a disturbance in pro-oxidant –antioxidant balance in favour of the former, leading to potential damage". Oxidative stress can result in adaptation or cell injury due to lipid peroxidation (Halliwell et al., 1995). Lipid peroxidation is a free radical mediated process. It is involved in the formation of lipid radicals, a rearrangement of the unsaturated lipids that results in a variety of degraded products like alkanes, MDA, conjugated dienes and lipid hydroperoxides and eventually cause damage to cells (Upsani et al., 2001). Exposure of cells to free radicals results in DNA damage causing mutagenesis, carcinogenesis and ultimately cell death, if antioxidant system is faulty. Increased accumulation of MDA and conjugated dienes in the cell can result in cellular degradation, biochemical and functional changes eventually leading to cell death (Cerutti, 1994).

Antioxidant enzymes such as SOD, CAT and GPX can directly counteract the oxidant attack, which may protect cells against LPO and DNA damage. SOD inhibits OH• production and has been shown to inhibit nuclear transcription factor in human cancer cell and it acts as an anti-proliferative agent. SOD also acts as an anti carcinogen and an inhibitor at initiation and promotion/transformation stages in carcinogenesis. CAT and Gpx are important antioxidant enzymes in the inactivation of many environmental mutagens. CAT is found to reduce the sister chromatid exchange levels. In cancer, CAT is found to be inactivated which may be due to high OH• production (Ray & Husain, 2002). SOD reacts with superoxide radicals and converts them to H<sub>2</sub>O<sub>2</sub>, excessive amount of these metabolites start lethal chain reaction which oxidises and disable structures that are required for cellular integrity and survival. Catalase catalyses the decomposition of hydrogen peroxide to water and oxygen. Reports suggested that oxygen free radicals and OH• directly affects and inactivates catalase (Koch et al.,

1989). Enhanced lipid peroxidation is associated with depletion of antioxidants. MDA is the major reactive aldehyde resulting from the peroxidation of biological membranes (Vaca et al., 1998). MDA is a secondary product of LPO, used as an indicator of tissue damage by a series of chain reaction (Ohkawa et al., 1979). MDA is mutagenic and a genotoxic agent that may contribute to the development of human cancer (Feron et al., 1991). LPO may directly induce DNA chain break and lipid peroxy and alkoxy radicals may cause base oxidation in DNA (Cochrane, 1991).

When Green tea polyphenols was administered to the 4-NQO induced animals, there was a significant reduction in the levels of LPO and OH• (Figure 1), which correlates with the study by Srinivasan et al. (2007). The previous studies on Green tea polyphenols states that aqueous solution of Green tea polyphenols was found to inhibit lipid peroxidation, scavenges OH• and superoxide radicals in vitro (Srinivasan et al., 2007). In the present study, administration of Green tea polyphenols for 7 days to group 4 (4-NQO-GTP) animals enhanced the activity of antioxidant enzymes (Figure 3) and decreased the levels of LPO and OH•, which could be due to the antioxidant activity of Green tea polyphenols. Catechin, one of the components of GTP, inhibits both LPO and OH• and significantly increased the activity of antioxidant enzymes (Gupta et al., 2002).

The cell is protected against damage by several mechanisms such as oxygen consumption, primary radical scavenging, release of bound endogenous reactants, inhibition of oxygen transport etc. (Koch et al., 1989). GSH plays a key role in protecting cells against free radicals and electrophiles and this could be due to the nucleophilicity of –SH group and due to the high reaction rate of thiols with free radicals. Figure 2 shows the effect of GTP on the levels of cellular thiols on the bone marrow of control and experimental animals. Among the protectors of oxidative stress and damage, cellular thiols are important, mainly GSH plays a major role thus, GSH belongs to the second line of antioxidant defense which is the most abundant non-protein thiol synthesized in vivo and serves as a scavenger of different free radicals (Ray & Husain, 2002).

Lipid peroxidation was also known to deplete the protein thiols. Casini et al. (1987) have shown that a significant decrease in protein thiols was observed only when lipid peroxidation is developed. Oxygen free radicals and hydrogen peroxide are able to react directly with protein sulphhydryl groups (Bellomo et al., 1983) and in the absence of glutathione this effect may become pronounced. Oxidative stress which can enhance s-thiolation cause's formation of one n mole protein SSG/n mole GSSG during t-butylhydroperoxide oxidation of mitochondria (Creighton, 1983). It was also found that during the conditions of oxidative stress, PSH were depleted. This was correlated with damage to the cells (Irshad & Chaudhuri, 2002).

In the present investigation, there was a decrease in GST in 4-NQO induced rats. Detoxification of 4-NQO is mainly carried out by GST, which catalyzes the nucleophilic substitution of the nitro group of 4-NQO

with thiols. Greater thiol loss under toxic and hypoxic conditions suggested oxidation by superoxide, peroxide or hydroxyl radical formed in the course of 4-NQO reductions (Varnes & Biaglow, 1979). Oxidative stress produced by 4-NQO reduction depletes thiol and inactivates GST, so detoxification of 4-NQO by conjugation lacks leading to accumulation of 4-NQO which then binds with DNA causing mutation. The activation of 4-NQO by phase I enzymes and detoxification and eliminates by phase II enzymes are in absolute balance that exist in cells and is an important determinant of whether exposure to carcinogens will result in toxicity and neoplasia. We assume that detoxification enzymes are functionally redundant and that only the simultaneous deficiency of several detoxification enzymes increases the risk for toxicity.

In the present study there was an increase in GST in simultaneous administration of GTP with 4-NQO. GTP transcriptionally activates phase II genes, thereby increasing the synthesis of antioxidant enzymes (Yu et al., 1997). So, elevation in the activity of GST enhances the detoxification of 4-NQO, thus the level of tissue damage was found to be decreased in rats simultaneously treated with GTP and 4-NQO.

MDA-DNA adducts are biomarkers of endogenous DNA damage. MDA is a naturally occurring endogenous product of lipid peroxidation and prostaglandin biosynthesis and binds covalently to proteins, RNA and DNA. The reaction with DNA is with deoxyguanosine giving rise to cyclic adducts, formed by the reaction of the carbonyl carbons of MDA at the N1 and N2 position of guanine. The major DNA adduct of MDA is a pyrimido purinone of deoxyguanosine, although adenine adducts are also formed (Zhang et al., 2002). A significant increase in the level of MDA-DNA adducts was observed in 4-NQO induced animals, this may be due to the production of MDA a highly reactive aldehyde which forms adduct with DNA. Treatment with GTP to 4-NQO induced animals depicted a significant decrease in the level of MDA-DNA adducts, this might be due to inhibition of oxidative stress by GTP, thereby reducing the production of MDA and ultimately DNA adducts which correlated with the previous studies by Isbrucker et al., (2006).

The results highlighted in this work depict the protective role of GTP extract in 4-NQO mediated oxidative stress in bone marrow toxicity, for the first time. These results shed adequate information to curtail a postulate in 4-NQO mediated toxicity. In future, work should be carried out to elucidate the role of 4-NQO in inducing cancers through bone marrow toxicity and the effect of GTP and its exact molecular mechanism inducing cancer.

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