Involvement of Wee1 in the Circadian Rhythm–Dependent Intestinal Damage Induced by Docetaxel

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ABSTRACT
Docetaxel, a semisynthetic taxane, is effective for the treatment of some solid cancers; however, docetaxel-induced intestinal damage leads to poor prognosis in some patients. Although such adverse effects have been reported to depend on the dosing-time of docetaxel, the mechanisms involved remain unclear. Wee1 expression is controlled by the clock gene complex, clock/bmal1, and contributes to cell-cycle progression. The present study was undertaken to evaluate the potential role of Wee1 in the circadian rhythm–dependent profile of docetaxel. Male mice were maintained under a 12-hour light/dark cycle. Intestinal damage after repeated dosing of docetaxel (20 mg/kg) for 3 weeks was more severe at 14 hours after light on (HALO) than at 2 HALO. The intestinal protein expressions of Wee1, phosphorylated CDK1, and cleaved Caspase-3 were higher in the 14-HALO group than in the 2-HALO group, whereas that of survivin was lower in the 14-HALO group. Thus, it is speculated that elevated Wee1 expression inhibited CDK1 activity more by phosphorylation, which in turn caused the lower expression of survivin and consequently more activated Caspase-3 in the 14-HALO group. There were no significant differences in plasma docetaxel concentrations between the 2- and 14-HALO groups. Bindings of CLOCK and BMAL1 to the E-box regions at the wee1 gene promoter were not altered by docetaxel treatment at 2 and 14 HALO. These findings suggest that Wee1 is directly or indirectly involved in the mechanism of the circadian rhythm–dependent changes in docetaxel-induced intestinal damage. However, the mechanism for a circadian rhythm–dependent profile of docetaxel remains to be determined.

Introduction
Docetaxel [(–)-(S,S)-2-benzoyloxy-5,20-epoxy-1,7,10-trihydroxy-11-ene-13-yl(2R,3S)-3-tert-butoxycarbonylamino-2-hydroxy-3-phenylpropionate trihydrate] is a semisynthetic taxane derived from the needles of the European yew (Taxus baccata). This agent binds to tubulin, leading to its polymerization, promotes microtubule assembly, and inhibits tubulin depolymerization (Pellegrini and Budman, 2005). The disruption of microtubules causes cell arrest at the G2-M phase of the cell cycle and subsequently leads to cell death by apoptosis or necrosis. Docetaxel causes cell death mainly by mitotic catastrophe (Morse et al., 2005; Fabbri et al., 2008), which is an aberrant form of mitosis and induces cell death by defects in mitosis (Roninson et al., 2001). Mitotic catastrophe, a process preceding apoptotic- or necrosis-induced cell death (Vakifahmetoglu et al., 2008), is controlled by numerous molecular factors such as cell-cycle–specific kinases (including cyclin-dependent kinase1 [CDK1], polo-like kinases, and aurora kinases), cell-cycle–checkpoint proteins, survivin, p53, caspases, and members of the Bcl-2 family (Castedo et al., 2004).

Many physiologic functions show circadian rhythms that are regulated by a biological clock system as follows (Mohawk et al., 2012). 1) A central clock in the suprachiasmatic nucleus influences peripheral clocks through hormonal and neural signals. 2) Individual peripheral tissues also have their own clock system and express a self-sustained circadian oscillation. 3) The clock system contains the following clock genes: clock, bmal1, per1-3, and cry1-2. Clock and Bmal1, the basic helix-loop-helix transcriptional activators, are positive regulators of the clock system, whereas Per1-3 and Cry1-2 are negative.

Although myelosuppression is generally a major dose-limiting toxicity for cancer chemotherapies, prophylactic use of granulocyte colony–stimulating factors and improved transplantation procedures ameliorate this adverse effect in many patients. On the other hand, nonhematologic toxic effects such as mucositis have now become the dose-limiting factor.

A recent study showed that cell cycle progression is under the influence of the circadian clock through the clock-controlled gene wee1 (Gerard and Goldbeter, 2012). Wee1 protein is a tyrosine kinase that selectively phosphorylates the Tyr15

ABBREVIATIONS: CDK1, cyclin-dependent kinase1; HALO, hours after light on; PCR, polymerase chain reaction; TUNEL, terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling.
residue of CDK1 and inactivates its activity (Chow and Poon, 2012; Magnussen et al., 2012), leading to G₂-M arrest.

The toxicity and efficacy of anticancer drugs have been shown to be improved by their optimal dosing time (Innominate et al., 2010). A previous animal study showed that the frequency of docetaxel-induced severe damage in the intestinal mucosa was greater after dosing at an active phase than at an inactive phase in mice (Tampellini et al., 1998). However, the underlying mechanism(s) of such dosing-time-dependent intestinal toxicity remains to be determined. This study was undertaken to evaluate the potential role of Wee1 in the chronotoxicological profile of docetaxel in mice.

Materials and Methods

Animals and Synchronization

Five-week-old male Balb/c mice (SLC, Hamamatsu, Japan) were maintained under a 12-hour light/dark cycle in a temperature-controlled room (23 ± 1°C) with food and water ad libitum. All mice were synchronized for more than 2 weeks before the initiation of the experiment. Time was expressed as hours after light on (HALO); 2, 6, and 10 HALO were during the light period when mice were commonly inactive, whereas 14, 18, and 22 HALO corresponded to the dark period when animals were commonly active. The experiment was approved by the Ethics Committee of Jichi Medical University (No. 12022; Tochigi, Japan) and performed in accordance with the Use and Care of Experimental Animals Committee of Jichi Medical University.

Preparation of the Dosing Solution

Docetaxel (Taxotere) was kindly supplied by Sanofi-Aventis (Tokyo, Japan). The drug was injected intraperitoneally. Docetaxel was dissolved in a solution to store 10 mg/ml and further diluted with saline just before each injection. The volume of the drug solution given to mice each time was 0.02 ml/g.

Evaluation of Intestinal Apoptosis

Docetaxel (0, 10, 20, 30, 40, 60, and 80 mg/kg per week) was given once a week for 3 weeks for mice. Because more than 30 mg/kg per week of the drug caused body weight loss in mice (Supplemental Fig. 1), 20 mg/kg per week of docetaxel was judged to be the maximum nontoxic dose. Docetaxel (20 mg/kg per week) was given to mice once a week for 3 weeks at one of the following different points (2, 10, 14, or 22 HALO). Seventy-two hours after the final dosing of the agent, the intestinal mucosa of the small intestine (proximal 8 cm) was removed, fixed in 20 N Mildform solution; Wako Pure Chemical Industries, Osaka, Japan), and embedded in paraffin blocks, and sections of 5 μm were put on glass slides. Apoptosis was detected using the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) method (Noda et al., 1998), using the Apop Tag Peroxidase In Situ Apoptosis Detection Kit (S7100; EMD Millipore, Billerica, MA). Specimens were dewaxed and immersed in phosphate-buffered saline for 5 minutes at room temperature, incubated with 20 μg/ml proteinase K for 15 minutes at room temperature, and then quenched of endogenous peroxidase in 2% hydrogen peroxide in phosphate-buffered saline. Terminal deoxynucleotidyl transferase enzyme was applied directly onto the specimens, which were then incubated at 37°C for 1 hour. The reaction was terminated by transferring the slides to stop/wash buffer for 10 minutes at room temperature, and then specimens were covered with peroxidase-conjugated antidigoxigenin antibody and incubated for 30 minutes at room temperature. Specimens were then soaked in staining buffer containing 0.05% dianaminobenzidine to achieve color development. Finally, the specimens were counterstained by immersion in Mayer’s hematoxylin solution. Apoptotic cells were counted under a light microscope in a good longitudinal crypt section. Starting at the base of the crypt column, the TUNEL-positive cells were counted up to the 18th cell position in each crypt. One hundred crypt sections were scored in each animal, and a frequency of TUNEL-positive cells per crypt was calculated (Ijiri and Potten, 1983). Dosing-time-dependent influence of docetaxel on intestinal apoptosis was also examined in female Balb/c mice.

Protein Extraction, Immunoprecipitation, and Western Blotting

Docetaxel (20 mg/kg) was given to mice once at 2 and 14 HALO. In a preliminary study, we measured an intestinal Wee1 protein expression level before and at 24, 48, and 72 hours after dosing of 20 mg/kg docetaxel. Wee1 protein expression level time dependently increased, reached to the highest at 48 hours and returned to the baseline at 72 hours after dosing. Therefore, the small intestine, containing epithelial cells and smooth muscles, was obtained 48 hours after dosing of the agent.

A small intestinal sample was homogenized in lysis buffer [50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Triton-X 100, 0.1% Noniident P-40, 4 mM EDTA, 4 mM NaF, 0.1 mM Na₃VO₄, 0.1 mM phenylmethylsulfonyl fluoride, and protease and phosphatase inhibitor cocktails (Nacalai Tesque, Kyoto, Japan)] and incubated on ice for 30 minutes. Insoluble debris was pelleted, and the protein concentration in the supernatant was determined using a BCA Protein Assay kit (Thermo Fisher Scientific, Rockford, IL).

For the analysis of Bax, cleaved Caspase-3, Wee1, survivin, and β-actin, lysate samples containing 20 μg of total protein were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was reacted with antibodies against Bax (1 μg/ml), Wee1 (1.5 μg/ml), survivin (1 μg/ml), β-actin (2 μg/ml) (Abcam, Cambridge, UK), or cleaved Caspase-3 (31 ng/ml) (Cell Signaling Technology, Danvers, MA). For the analysis of CDK1 phosphorylation [p-CDK1 (Tyr 15)] and CDK1, intestine lysate (1.8-mg protein) was immunoprecipitated with an anti-CDK1 antibody (Santa Cruz Biotechnology, Dallas, TX) at 4°C overnight. Immunocomplexes were captured with protein A-agarose (Roche Applied Science, Indianapolis, IN), and samples were allowed to mix at 4°C for 2 hours. Immunoprecipitates were washed and then separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Subsequently, the membrane was reacted with an anti-p-CDK1 (Tyr 15) (2 μg/ml) or anti-CDK1 (2 μg/ml) antibody (Santa Cruz Biotechnology). Specific antigen-antibody complexes were visualized using horseradish peroxidase–conjugated secondary antibodies and an ECL Plus detection kit (GE Healthcare, Buckinghamshire, UK).

RNA Extraction and Real-Time Polymerase Chain Reaction Analysis.

Docetaxel (20 mg/kg) was given to mice once at one of the following six different points (2, 6, 10, 14, 18, or 22 HALO), and the small intestine containing epithelial cells and smooth muscles was obtained 48 hours after dosing of the agent. Intestinal total RNA was isolated using an RNasy Mini Kit (Qiagen, Valencia, CA), and reverse transcription was performed using the PrimeScript RT reagent kit (Takara, Shiga, Japan). Reverse transcription products were subjected to PCR amplification using the primers shown in Table 1.

Table 1

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Primer Sequence</th>
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<tr>
<td>Gapdh</td>
<td>5'-TTGGTGCTCGGTCGATGCTGA-3'</td>
</tr>
<tr>
<td>Wee1</td>
<td>5'-TTGTGGTGTGAGTCCGGAGG-3'</td>
</tr>
<tr>
<td>Bmal1</td>
<td>5'-CGGTTGCTCACTGAACTGCCATC-3'</td>
</tr>
<tr>
<td>Clock</td>
<td>5'-AACCCTGACGGGTTAGCTGGAATG-3'</td>
</tr>
</tbody>
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Table 1: Sequences of primers for real-time PCR analysis.
Chromatin Immunoprecipitation and Real-Time Polymerase Chain Reaction Analysis

Docetaxel (20 mg/kg) was given to mice once at 2 and 14 HALO, and the small intestine, containing epithelial cells and smooth muscles was obtained 48 hours after dosing of the agent. The intestine was fixed with 1% formaldehyde at room temperature for 15 minutes. The sample was homogenized, and cross-linked chromatin was sonicated on ice. Chromatin immunoprecipitation (ChIP) was performed using Dynabeads Protein G (Invitrogen), and a fragmented chromatin was incubated with the antibody against CLOCK (Abcam) and BMAL1 (Novus Biologicals, Littleton, CO). DNA was purified and amplified using real-time polymerase chain reaction (PCR) for the surrounding E-boxes in the promoter region of the *wee1* gene. The sequences of primers and probes for amplification were described in Table 2.

Plasma Docetaxel Concentrations

Docetaxel (20 mg/kg) was given to mice once at 2 and 14 HALO. Blood was collected 0.5, 1, 2, 4, and 8 hours after the injection and was centrifuged. Sample extraction was performed as described (Vergniol et al., 1992) with minor modifications. Plasma sample (150 µl) spiked with 10 µl of an internal standard solution (paclitaxel 5 mg/ml) was mixed with 250 ml of water. This aqueous solution was loaded onto a C2 Bond Elute microcolumn (Varian, Harbor City, CA), which was preactivated with 1 ml of methanol and 1 ml of water. A wash step was performed using 1 ml of water and 1 ml of 5% methanol. The cartridge was removed to another collection tube, and the sample was eluted using 120 µl of methanol. The eluate was directly applied to high-performance liquid chromatography (HPLC) analysis.

The HPLC system consisted of a liquid pump (PU-2080Plus; JASCO, Tokyo, Japan), a degasser (DG-2080-54; JASCO), an autosampler (AS-2059Plus; JASCO), and a UV detector (UV-2070Plus; JASCO). Control of the HPLC system and data collection was performed by ChromNAV (JASCO). The column was a Mightysil RP-18 (4.6 × 250 mm, 5 µm; KANTO Chemical Co., Inc., Tokyo, Japan) fitted with a RP-18 guard column (4.6 × 5 mm, 5 µm; KANTO Chemical). The mobile phase contained methanol and 0.3% orthophosphoric acid (73:27), its flow rate was 1.0 ml/min, and UV detection was used at 225 nm. Calibration curves (using peak height ratios) were linear over the range 100–5000 ng/ml (r² = 0.997). The coefficients of variation were 3.2% (intraday) and 5.7% (interday).

Statistical Analysis

Groups were compared by one-way or two-way ANOVA, and the difference between the two groups was analyzed by the Bonferroni-Dunn test. Daily rhythmicity was analyzed by the Cosinor method and one-way ANOVA. P < 0.05 was considered to be significant.

Results

Dosing-Time–Dependent Influence on Docetaxel-Induced Apoptosis in the Mouse Intestine

Docetaxel (20 mg/kg) or vehicle was given once a week for 3 weeks to mice. At the end of the trial, apoptotic bodies in the intestine crypt significantly increased with docetaxel in the 2-, 10-, and 14-HALO groups (Fig. 1). The variability in the docetaxel-treated 14-HALO group was significantly greater than that of the drug-treated 2-HALO group. In female mice, the docetaxel-induced intestinal apoptosis in the 14-HALO group was also significantly greater than that in the 2-HALO group (Supplemental Fig. 2).

Dosing-Time–Dependent Influence of Docetaxel on Bax, Cleaved Caspase-3, Wee1, CDK1 Phosphorylation, and Survivin in the Mouse Intestine

To evaluate the potential molecular mechanism involved in the chronotoxicological profile of docetaxel, the expressions of Bax, cleaved Caspase-3, Wee1, CDK1, and survivin were determined 48 hours after repeated dosing of the agent at 2 and 14 HALO (Figs. 2 and 3).

The expressions of Wee1 and phosphorylated CKD1 were significantly elevated by docetaxel in the 2-HALO group, but not in the 14-HALO group. On the other hand, cleaved Caspase-3 expression was significantly elevated by docetaxel in the 14-HALO group, but not in the 2-HALO group.

The expressions of Wee1 and phosphorylated CKD1 were significantly elevated after dosing of docetaxel at 14 HALO, but not at 2 HALO. In addition, docetaxel significantly reduced
survivin expression in the 14-HALO group but not in the 2-HALO group. The survivin expression level in the docetaxel-treated 14-HALO group was significantly smaller than that in the drug-treated 2-HALO group.

**Dosing-Time–Dependent Influence of Docetaxel on Wee1 and Clock Gene mRNA Expressions in the Mouse Intestine**

The expression of Wee1 mRNA in the vehicle-treated group showed a significant daily rhythm with a peak at 14 HALO ($P < 0.01$ by Cosinor and $P < 0.01$ by ANOVA). Docetaxel significantly elevated its expression after dosing at 14 HALO, and its expression level in the docetaxel-treated 14-HALO group was greater than that in the drug-treated 2-HALO group (Fig. 4).

Bmal1 mRNA expression in the vehicle-treated group also showed a significant daily rhythm with a peak at 22 HALO ($P < 0.01$ by Cosinor and $P < 0.01$ by ANOVA), whereas Clock

**Fig. 2.** Dosing-time–dependent influence of docetaxel on apoptosis-related proteins in the mouse intestine. Representative images of Western blot were shown in the top panel. The mean value of the vehicle group at 2 HALO was set to be 1.0. Vehicle (open bar), docetaxel (DOC; gray bar), mean ± S.E., $n = 6–7$ in each, *$P < 0.05$ versus vehicle.

**Fig. 3.** Dosing-time–dependent influence of docetaxel on the G2-M cell-cycle (A) and survivin (B) protein expressions in the mouse intestine. Representative images of Western blot were shown in the top panel. The mean value of the vehicle group at 2 HALO was set to be 1.0. Vehicle (open bar), docetaxel (DOC; gray bar), mean ± S.E., $n = 6–7$ in each in A, $n = 8$ in each in B, *$P < 0.05$ versus vehicle, **$P < 0.01$ versus 2 HALO.
mRNA expression did not. Docetaxel did not change the expressions of these clock genes.

**ChIP Analysis at the wee1 Gene Promoter in the Mouse Intestine**

ChIP analysis at the wee1 gene promoter in the mouse intestine was performed 48 hours after dosing of docetaxel. The bindings of CLOCK and BMAL1 to E-box regions were not altered by the docetaxel treatment at 2 and 14 HALO (Fig. 5).

**Chronopharmacokinetic Study of Docetaxel**

Plasma docetaxel concentrations were measured after dosing of the agent (20 mg/kg) at 2 and 14 HALO. Plasma drug concentrations were not detected at 8 hours in both groups. There were no significant differences in plasma docetaxel concentrations between the 2- and 14-HALO groups at any sampling points (Fig. 6).

**Discussion**

Docetaxel is effective in the treatment of some solid cancers such as breast, lung, ovarian, and prostate cancers and improves the survival of these patients (Montero et al., 2005). However, mucositis is one of the dose-limiting toxic effects of docetaxel and leads to poor prognosis in some patients (Ibrahim et al., 2000). Therefore, a safer regimen is needed to ameliorate this drug-induced adverse effect. Chronotherapy is one of the approaches used to address this issue. The frequency of docetaxel-induced severe damage in the intestinal mucosa has been reported to be greater after dosing at an active phase than that at an inactive phase in mice (Tampellini et al., 1998). The present study also provided results to indicate that docetaxel-induced intestinal damage is greater during repeated dosing at an active phase in mice.

The mammalian cell cycle network is coupled to the circadian clock, *clock/bmal1*, through circadian variations in the Wee1 protein (Gerard and Goldbeter, 2012). A previous study using the small intestine in mice showed that the mRNA expressions of Bmal1 and Wee1 exhibited circadian variations with peaks at the late dark period and early dark period, respectively (Polidarova et al., 2009), which are similar to the present findings. In addition, this study showed for the first time that repeated treatment with docetaxel elevated Wee1 mRNA expression, especially at the early dark period (14 HALO).

Wee1 has been reported to inhibit CDK1 activity by phosphorylation (Chow and Poon, 2012; Magnussen et al., 2012), which in turn decreases survivin (Chen et al., 2013) and consequently activates Caspase-3 (Jones et al., 2010; Rubio et al., 2012). The phosphorylation of CDK1 and decrease in survivin are also involved in the mechanism of apoptosis via mitotic catastrophe (Castedo et al., 2004; Lamers et al., 2011). In this study, the following results were obtained after repeated dosing of docetaxel at 14 HALO: The intestinal protein expressions of Wee1 and phosphorylated CDK1 increased and that of survivin decreased. In addition, intestinal cleaved-Caspase-3 expression was elevated in the 14-HALO group. However, these docetaxel-related changes were relatively small in the 2-HALO group. On the basis of

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**Fig. 4.** Relative mRNA expressions of Wee1 (A) and clock genes (B) in the mouse intestine 48 hours after dosing of docetaxel at six different time points. The mean value of the vehicle group at 2 HALO was set to be 1.0. Vehicle (open bar), docetaxel (gray bar), mean ± S.E., n = 5–7 in each in A, n = 4 in each in B. **P < 0.01 versus vehicle, ## P < 0.01 versus 2 HALO (A); # P < 0.05 and ### P < 0.01 versus 22 HALO (B).
these findings, the Wee1-CDK1-survivin-Caspase-3 pathway is thought to be involved in the mechanism of dosing-time–dependent changes in docetaxel-induced intestinal damage (Fig. 7). Docetaxel has multiple anticancer mechanisms, including the upregulation of Bax (Mhaidat et al., 2007; Ye et al., 2012). In this study, intestinal Bax expression was significantly elevated in the 2-HALO group after repeated dosing of docetaxel. Therefore, the Bax-related pathway may have been involved in the drug-induced intestinal damage observed in this study. However, this pathway may not play a major role in the dosing-time–dependent intestinal damage induced by docetaxel.

Repeated dosing of docetaxel elevated the intestinal mRNA expression of Wee1, especially at 14 HALO, but did not change those of Bmal1 and Clock in this study. The transcription of wee1 is directly regulated through the binding of Clock-Bmal1 heterodimers to E-box sequences in the promoter region (Matsuo et al., 2003). Therefore, to evaluate the potential mechanism, the dosing-time–dependent influence of docetaxel on the binding of CLOCK and BMAL1 to the E-box regions at the wee1 gene promoter was examined in 2- and 14-HALO groups. Because the bindings of these proteins to E-box regions were not altered by repeated dosing of docetaxel at 2 and 14 HALO, such a mechanism may not be involved in the dosing-time–dependent influence on intestinal Wee1 expression. Dosing-time has been reported to influence the blood concentrations of many drugs and consequently their effects or adverse effects (Lemmer, 2000). In this study, no significant differences were observed in plasma docetaxel concentrations between the 2- and 14-HALO groups. Therefore, chronopharmacokinetic differences may not be involved in the mechanism of the dosing-time–dependent influence of docetaxel on intestinal Wee1 expression. Further study is needed to evaluate the mechanism(s) of this chronotoxicological event.

The present and previous (Montero et al., 2005) studies showed that docetaxel-induced intestinal damage was less severe after dosing of the agent during the light period than during the dark period in nocturnal rodents. In addition, the antitumor effect of docetaxel was also reported to be enhanced during repeated dosing at the light period (Montero et al., 2005). Our preliminary study showed that the antitumor effect of docetaxel after dosing at 2 HALO was similar to that after dosing at 14 HALO (data not shown). On the basis of these findings in nocturnal rodents, we suggest that repeated dosing of docetaxel at night is a safer regimen for ameliorating drug-induced intestinal damage in human cancer patients. It is also anticipated that dosing at night may not diminish the antitumor effect of docetaxel. Clinical studies with cancer patients are needed to confirm these hypotheses.


Obi-Ioka et al. (2013) Docetaxel-induced intestinal damage was greater after dosing at 14 HALO than at 2 HALO.

**Authorship Contributions**

**Conducted experiments:** Obi, Usukawa, Ishikawa-Kobayashi.

**Contributed new reagents or analytic tools:** Obi.

**Performed data analysis:** Obi, Usukawa.

**Wrote or contributed to the writing of the manuscript:** Usukawa, Kusama, Fujimura.

**References**


Supplemental Figure 1

Body weight change after dosing of docetaxel at 2HALO or 14HALO

Arrows represent the drug-dosing.

- × vehicle, 〇 10 mg/kg, ● 20 mg/kg, △ 30 mg/kg, ▲ 40 mg/kg, ○ 60 mg/kg,
- ◆ 80 mg/kg

Mean ± SE, n=5-8 in each
Supplemental Figure 2
Dosing-time dependent influence of docetaxel on apoptosis in the female mice intestine

Docetaxel (20 mg/kg/week) was given to female mice once a week for 3 weeks at 2 or 14 HALO. Seventy two hrs after the final dosing of the agent, the intestinal mucosa of small intestine (proximal 8 cm) was removed. The procedure of TUNEL method was described in the MATERIALS and METHODS section.

* p< 0.05 and ** p< 0.01 vs. vehicle, # p<0.05 vs. 2 HALO