



## **In vivo bioassay to detect irinotecan-stabilized DNA/topoisomerase I complexes in rats**

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**Technical Report** ((4073 words))

**In vivo bioassay to detect irinotecan-stabilized DNA/topoisomerase I complexes in rats**

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**Keywords:** methods, topoisomerase poison, cleavable complex, DNA strand breaks, comet assay.

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**Abbreviations:** bw, body weight; ICE, *in vivo* complexes of enzyme to DNA; TI, tail intensity; T/C, test over control.

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## Abstract

Irinotecan is an anticancer agent stabilizing topoisomerase I/DNA complexes. No test system has been reported so far allowing to directly determining irinotecan-induced stabilization of topoisomerase I/DNA complexes in organs *in vivo*. We adapted a in vivo complexes of enzyme to DNA (ICE) bioassay to assess irinotecan activity in the stomach, duodenum, colon and liver of male Wistar rats after a single treatment with irinotecan (100 mg/kg body weight, intraperitoneally) compared to the control group receiving 0.9% sodium chloride intraperitoneally. In addition, the DNA strand breaking properties of irinotecan were measured in mucosa cells from the distal colon by single cell gel electrophoresis (comet assay) to investigate the association of topoisomerase poisoning and DNA damage *in vivo*. A single dose of irinotecan significantly increased amounts of topoisomerase I covalently bound to DNA in stomach, duodenum, colon and liver. Concomitantly, the irinotecan-treated group showed significantly higher amounts of DNA strand breaks in colon mucosa cells compared to the control group. The ICE-bioassay and the comet assay represent two test systems for investigating the impact of topoisomerase I poisons on DNA-integrity in colon tissues of Wistar rats.

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Introduction

The camptothecin analogue irinotecan ((7-ethyl-10-(4-[1-piperidino]-1-piperidino)-carbonyloxy-camptothecin) is an anticancer drug used in the therapy of colorectal cancer [1-2]. It is a semi-synthetic pro-drug with improved water solubility in comparison to camptothecin [3, 4]. Irinotecan is cleaved by carboxylesterases to form the active metabolite 7-ethyl-10-hydroxycamptothecin (SN-38), which possesses a 100 to 1,000 times higher cytotoxic capacity than the parent drug [2, 5].

Camptothecin and its analogues represent topoisomerase I poisons. Topoisomerase I is a monomeric enzyme of 100 kDa constitutively expressed throughout the cell cycle in eukaryotic cells [6]. Malignant cells express higher levels of topoisomerase I in comparison to their non transformed counterparts. Moreover, significant differences in topoisomerase I levels have been shown between several tumor types [7, 8].

Topoisomerase I introduces a transient single strand break in the DNA double helix by forming a covalent DNA-topoisomerase intermediate, the so-called cleavable complex. Torsion stress is released by strand rotation, and religation of the cleaved strand. Low levels of topoisomerase linked to DNA are tolerated by the cells. However, camptothecin analogues increase the concentration of cleavable complexes, causing DNA double strand breaks resulting in chromosomal aberrations, sister chromatide exchange and cell death [5, 9, 10].

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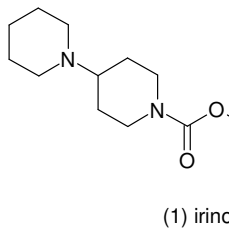
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Topoisomerases are important cellular targets for the development of chemotherapeutics. Detection systems for covalent binding of topoisomerase to DNA and the contribution of the DNA/topoisomerase intermediate to DNA-damage *in vivo* are rare but are important tools investigating the underlying mechanism of action of topoisomerase inhibitors. The *in vivo* complexes of enzyme to DNA (ICE)-bioassay represents a common technique detecting the level of topoisomerase covalently bound to DNA *in vitro* [11, 12]. This assay is specific for the different topoisomerase isoforms due to the employment of respective antibodies. Several studies have demonstrated that camptothecin stabilizes the cleavable complex in tumor cell lines, [13-15]. Topoisomerase II (etoposide) and topoisomerase I (topotecan) poisons have been reported to exhibit therapeutic activity in Non-Hodgkin lymphoma characterized as topoisomerase/DNA complex formation in the blood using the ICE-bioassay [16]. In a phase II clinical trial treatment with topotecan increased topoisomerase I/DNA intermediates in the peripheral blood of the patients with Non-Hodgkin lymphoma and melanoma [17]. However, whether the ICE-bioassay is applicable in assessing the ability of camptothecin analogues to increase levels of topoisomerase I/DNA intermediates in organs of animals treated with topoisomerase I poisons, has not been studied so far. Therefore the goal of the present study was to investigate whether the ICE-bioassay is a sensitive test system for the detection of topoisomerase I/DNA-complexes in stomach, duodenum,

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colon and liver of Wistar rats treated with a single dose of irinotecan.

Further, the stabilization of the cleavable complexes caused by irinotecan has been correlated with its DNA strand breaking properties as determined by comet-assay.

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**Materials and methods**

**Animals.** Male Wistar rats (n = 18; 100 g body; Harlan Winkelmann, Borchten, Germany) were housed in a temperature and humidity-controlled animal facility under ambient temperature of 21 ± 2 °C and a 12 h-12 h light-dark cycle. After one week of adaptation, the rats of the pilot and the main study were allowed consuming *ad libitum* tap water and a standard experimental diet (AIN93G, Ssniff, Soest, Germany). The entire study has been approved by the ethics committee responsible for the administrative district of Karlsruhe (35-9185.81/G-88/06).

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**Experimental protocol.**

**Pilot study:** One week after arrival, the rats (n = 12) were randomly assigned to a treatment group (n = 6) receiving intraperitoneal injections of irinotecan hydrochloride x 3H<sub>2</sub>O [50 mg/kg body weight] (Campto®, Pfizer, Karlsruhe, Germany; 20 mg/mL) every 24 h for 3 days. In parallel, the control group (n = 6) received 0.9% sodium chloride intraperitoneally.

**Main study:** One week after arrival, the rats (n = 18) were randomly assigned to a treatment group (n = 9) receiving a single intraperitoneal

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injection of irinotecan hydrochloride x 3H<sub>2</sub>O [100 mg/kg body weight] (Campto<sup>®</sup>, Pfizer, Karlsruhe, Germany; 20 mg/mL) and to a control group (n = 9) receiving 0.9% sodium chloride intraperitoneally.

24 h (pilot study) or rather 4h (main study) after the injection, animals were sacrificed by decapitation and biological samples (liver, stomach, duodenum, and colon) were obtained and processed as detailed below for the respective methods.

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### **In vivo complexes to enzyme bioassay (ICE-Assay)**

Immediately after decapitation of the animals, liver, stomach, duodenum and the colon were rapidly removed. After separation of the colon into the proximal (see comet assay) and the distal portion, the latter part and all other removed tissue were transferred into a 50 mL tube filled with

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phosphate buffered saline. The organs were immediately cut into small pieces with a scalpel and homogenized in 10 mL N-laurylsarcosine-containing (1% w/v) TE-buffer (10 mM Tris/HCl (pH 8.0), 1 mM EDTA).

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The viscous DNA-containing lysates were sheared with an 18-gauge needle and stored at -80 °C. The ICE-bioassay was performed in accordance to a modified method of Subramanian *et al.* [11]. Cell lysates

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were layered onto a cesium chloride gradient in polyallomer tubes (Beckman Coulter GmbH, Krefeld, Germany). Cesium chloride solutions with densities of 1.82, 1.72, 1.5 and 1.37 were prepared in TE-buffer (10 mM Tris/HCl, pH 8.0, 1 mM EDTA). The gradient consisted of four layers



cesium chloride with a decreasing density from the bottom to the top. The tubes were centrifuged at 100,000 x g for 24 h at 20 °C. 20 fractions (300 µL) were collected from the bottom of the tubes. The DNA concentration in each fraction was measured at 260 nm (NanoDrop, Peqlab Biotechnologie, Erlangen, Germany).

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All fractions were diluted with the equivalent volume of 25 mM sodium phosphate buffer pH 6.5 blotted onto a nitrocellulose membrane using a slot blot apparatus (Minifold II, Whatman®/Schleicher & Schuell, Dassel, Germany). After blocking the membrane with TBST-buffer (20 mM Tris/HCl, pH 7.5, 137 mM sodium chloride und 0.1% v/v Tween-20) for 3 h at room temperature the covalent complexes formed between human topoisomerase and DNA as well as free topoisomerase were detected using rabbit polyclonal antibodies specific for either topoisomerase I (100 kDa), topoisomerase IIα (170 kDa) or topoisomerase IIβ (180 kDa) at a 1:250 or 1:500 dilution, respectively. An anti-rabbit IgG peroxidase conjugate (1:2,000) was used as secondary antibody. All antibodies were purchased from Santa Cruz Biotechnology, Heidelberg, Germany. The respective chemoluminescent signals (Santa Cruz Biotechnology, Heidelberg, Germany) were analyzed using the LAS 3000 imager with the AIDA Image Analyzer 3.52 software for quantification (Raytest, Straubenhardt, Germany). Signals of corresponding fractions in treated and control animals were plotted as the mean ratio of arbitrary light units

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(test over colon control [%]). In the calculation of the T/C [%] values; DNA concentration has been respected.

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### Single cell microgel electrophoresis assay (Comet assay)

The proximal part of the colon was placed in 37 °C HBSS (137 mM sodium chloride, 5.4 mM potassium chloride, 337 µM disodium hydrogen orthophosphate, 278 µM dipotassium hydrogen orthophosphate, 5.6 mM glucose, 4.2 mM sodium bicarbonate, 20 mM HEPES). Subsequently, mucosa cells from the colon sample were isolated and electrophoretically processed according to the protocol published by Pool-Zobel *et al.* [18].

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Deleted: About 20 µL of cell suspension ( $2 \times 10^5$  cells) was mixed with 75 µL low melting agarose and placed between two layers of agarose on a glass slide. Three slides per animal were placed in lysis buffer (100 mM EDTA, 1% Triton X 100, 2.5 mM sodium chloride, 1% lauroyl sarcosine sodium salt, 10 mM Tris, 10% DMSO) for at least 1 h. Then, slides were placed in an electrophoresis chamber containing alkaline buffer (1 mM EDTA, 300 mM sodium hydroxide) for 20 min to allow DNA unwinding. Electrophoresis was carried out at 25 V, 300 mA for 20 min.

Fifty cells of each slide were analyzed using the imaging software of Perceptive Instruments (Halstead, UK). The amount of damaged DNA was expressed as the percentage of DNA in the tail (tail intensity, TI).

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### Statistical analysis

All data are presented as the mean  $\pm$  standard error of the mean (SEM). All statistical calculations were performed with the Microcal Origin program (OriginLab Corporation, Northampton, USA). Statistical analysis was performed by unpaired Student's *t* test

## Results

### Body and organ weights

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The average body weight and the organ weights of the irinotecan groups in the pilot and main studies did not significantly differ from the respective irinotecan groups (Table 1) except of the duodenum weight which was statistically reduced in the irinotecan group.

Topoisomerase I poisoning

General observations/specificity testing

The lysates prepared from stomach, duodenum and distal colon were separated into free protein (fractions 10-20) and protein/DNA-complexes (fraction 1-9, Figure 1A). In the liver lysate the highest DNA amount was measured in the fractions 8-13, thus indicating the relevance to analyze the DNA amount of each fraction identifying the DNA-containing fractions.

As shown in the immunoblot (Figure 1B) using polyclonal antibodies specific for topoisomerase I, II $\alpha$  and II $\beta$  IgG, only topoisomerase I/DNA complexes were detected in the DNA peak fractions as shown in a representative colon sample of an irinotecan treated animal. To characterize the specificity of the polyclonal antibody to solely detect topoisomerase I, we have used rats treated with the topoisomerase II poison etoposide (20 mg/kg body weight). No etoposide stabilized topoisomerase II/DNA intermediates were detected with the topoisomerase I antibody thus indicating a lack of crossreactivity with topoisomerase II isoform.

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The average body weight as well as the organ weights of irinotecan treated animals (3 times 50 mg/kg body weight intraperitoneally every 24 hours, Table 1) were found to be lower compared to the control group, but only the duodenum weight was significantly decreased. ¶  
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**Pilot study:**

After repeated daily injection of irinotecan over three days levels of topoisomerase I covalently bound to DNA increased in the duodenum, colon and liver of irinotecan-treated animals compared to controls (Figure 2 A, B). However, only in the colon this increase was found to be significant. No difference in the status of topoisomerase I/DNA complexes in the stomach between the irinotecan and the respective control group was observed (Figure 2 A, B). Furthermore, the control group exhibited a high amount of cleavage complexes in the duodenum compared to the colon and the liver (Figure 2B).

**Main study**

A single dose of irinotecan was associated with a significant increase of topoisomerase I covalently bound to the DNA in stomach ( $p < 0.001$ ), duodenum ( $p < 0.01$ ), colon ( $p < 0.001$ ) and liver ( $p < 0.001$ ) in comparison to respective controls (Figure 2C, D). Levels of DNA/topoisomerase I intermediates in the colon of irinotecan-treated rats was 5-fold higher compared to controls while cleavable complexes in duodenum increased 4-fold, in stomach 3.5 fold and liver 2.5 fold (Figure 1D). The stabilization of the cleavable complex was most pronounced in the colon followed by the stomach, liver and the duodenum with lower amounts of topoisomerase I covalently linked to DNA.

**DNA-strand breaking effects**

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In the main study, DNA damage in mucosal cells of the proximal colon of controls exhibited low levels of DNA-strand breaks as detected by comet assay (TI =  $3.8 \pm 0.6$  %, data not shown). The single treatment with irinotecan significantly increased DNA strand breaks (TI =  $11.7 \pm 0.5$  %) compared to controls. In contrast, animals receiving three daily injections of Irinotecan in frame of the pilot experiment exhibited no enhanced DNA damage compared to controls (data not shown).

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Discussion

The study has shown that the topoisomerase I poison irinotecan stabilizes covalent topoisomerase I/DNA complexes in stomach, duodenum, colon and liver of Wistar rats. The amount of topoisomerase bound to DNA was measured using the ICE-bioassay which is based on the separation and quantification of free topoisomerase I and covalent topoisomerase I/DNA complexes. We could demonstrate that irinotecan specifically reacted as a topoisomerase I poison. These *in vivo* results are in line with earlier *in vitro* studies showing that in ICE assays using HeLa cells exposed to the topoisomerase I poison topotecan a strong signal with a topoisomerase I antibody was obtained but none with a topoisomerase II antibody [16]. In the pilot study, rats treated intraperitoneally with daily doses of irinotecan exhibited significantly enhanced levels of topoisomerase I/DNA intermediates only in the colon, 24 h after the terminal injection. In patients with Non-Hodgkin lymphoma a significant increase of topoisomerase

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I/DNA complex formation during the infusion of topotecan with a rapid decrease within 30 min after drug removal has been reported [16].

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Furthermore, *in vitro* studies have demonstrated that covalent topoisomerase I/DNA intermediates are potently degraded by the 26S proteasome [19-21]. Therefore a proteasome-mediated degradation of the cleavable complex 24 h after the final irinotecan application needs to be considered. Furthermore, animals of the control group exhibited a high

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level of topoisomerase I/DNA complexes in stomach and duodenum. The high background status of cleavable complexes in the control group as

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well as the tendency of reduced organ and body weight of animals receiving repeated doses of irinotecan might reflect technical limitations of this study design. This is inline with the observation, that no irinotecan enhanced DNA-damage could be detected in colon mucosal cells of repeatedly treated rats 24 h after the final treatment (data not shown).

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After 24 h an effective repair of enzyme/DNA-intermediates or DNA damage initially induced by topoisomerase poisons can be expected. For example, the enzyme tyrosyl-DNA-phosphodiesterase 1 has been implicated in the efficient repair of irreversible topoisomerase/DNA complexes [22]. Furthermore, tyrosyl-DNA-phosphodiesterase 1 has also been shown to be a part of the base excision repair complex [22, 23].

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Based on these observation of the pilot study the experimental design has been modified using a single treatment and analysis of samples 4 h after the irinotecan injection as a maximal time of 3 hours to transfer the

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prodrug irinotecan to the respective metabolite SN-38 was assumed [24, 25]. Further, own studies have shown topoisomerase poison induced cleavable complex stabilization *in vitro* after one hour of incubation [26]. In the investigated organs of irinotecan-treated rats a significant increase of topoisomerase I covalently bound to DNA was observed, compared to controls.

Although the JCE-bioassay represents a useful technique to study the activity and function of topoisomerase inhibitors in biological systems, data about its use *in vivo* are very limited so far. Subramanian *et al.* [17] and Kancherla *et al.* [16] showed that cleavable complex formation correlates with the response to topoisomerase poisons in patients with Non-Hodgkin lymphoma. The JCE-bioassay is a well established test system to investigate the mechanism of action of topoisomerase inhibitors *in vitro*.

Classical tumor therapeutics e.g. camptothecin [13], topotecan [11, 16] or etoposide [12], were characterized as topoisomerase poisons stabilizing the cleavable complex in cell culture experiments. To our knowledge, the present study is the first investigation of topoisomerase covalently linked to DNA in rodent organs and further demonstrating a significant increase after a single injection of irinotecan. It has been reported that humans show certain variability in the response to topoisomerase poisons indicating a variance in cleavable complex stabilization or degradation/repair [16]. Among other factors, this variance might be caused by different expression levels of topoisomerase I, by differences in

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the enzymatic activation of the pro-drug to its metabolite SN-38 by carboxylesterases in the liver and/or by the inactivation by uridine-diphosphate glucuronosyltransferase 1A1 [1, 16, 17, 27].

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Furthermore we showed in the main study that the stabilization of the topoisomerase I/DNA intermediates is associated with a significant increase of DNA strand breaks in the colon. In line with our results are reports of enhanced damage of DNA in whole blood, bone marrow, thymus, liver, and in the distal part of the intestine as reported in rats treated with the topoisomerase II poison etoposide [28]. Gibson *et al* [29] reported that irinotecan increased apoptosis in the small and large intestine in rats with implanted breast cancer.

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Summarizing our data, the ICE-bioassay and the comet assay are methods valid for identifying target organs of the topoisomerase I poison irinotecan *in vivo*. So far only indirect effects of topoisomerase poisons *in vivo* e.g. cytotoxicity or apoptosis induction as well as pharmacokinetic studies have been reported [29-31]. The advantage of the ICE-bioassay in combination with the comet assay is the direct detection of the cleavable complex in respective target tissues in parallel to the level of DNA strand breaks. The combination of these methods represents a technical progress to analyze the direct mechanism of action of topoisomerase poisons *in vivo*, being a useful tool to further clarify topoisomerase poisoning of cancer therapeutics as well as plant or food constituents e.g.

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genistein or quercetin [12] relevant for a comprehensive risk/benefit  
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**Acknowledgement**

The study was performed as a part of the FlavoNet, supported by the grants MA1659/6-1/2 and WA 1587/1-1 of the Deutsche Forschungsgemeinschaft (DFG).  
“The authors have declared no conflict of interest”.

For Peer Review

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Figure legends

**Figure 1:** Experiments for validation of the *in vivo* complex to enzyme (ICE) bioassay quantifying levels of topoisomerase I/DNA complexes in selected organs of Wistar rats treated with a single dose of irinotecan. (A) DNA-gradient of lysates prepared from stomach, duodenum, colon and liver of a representative irinotecan- treated animal. (B) Immunoblot of a colon sample derived from a representative irinotecan-treated animal.

**Figure 2:** The ICE-bioassay was used for the detection of topoisomerase I/DNA intermediates in stomach, small intestine, colon and liver of Wistar rats used for the pilot study (A,B) and in the main study (C, D). The data presented in A and C are the mean  $\pm$  SEM (pilot study: n =6 animal/group; main study: n=9 animals/group). (B, D) Immunoblot of representative experiments: a, control group; b, irinotecan group. The indicated *p*-values (Student's *t* test) were referred to the respective control group.

**Deleted: Figure 1:** Preliminary tests to validate the *in vivo* complex to enzyme (ICE) bioassay to quantify the levels of topoisomerase I/DNA complexes in selected organs of Wistar rats treated with a single dose of the topoisomerase I poison irinotecan. hydrochloride x 3 H<sub>2</sub>O (100 mg/kg body weight).

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**Deleted:** The lysates of stomach, duodenum, colon or liver of the treatment group and the control group were fractionated by a cesium chloride gradient and the single fractions (n = 20) were blotted onto a nitrocellulose membrane. Immunoblots were probed with a polyclonal antibody against either topoisomerase I, topoisomerase II $\alpha$  or topoisomerase II $\beta$ , respectively. The DNA content of the fractions was measured with a nanodrop spectrophotometer at 260 nm. (A) DNA-gradient of a representative example for stomach, duodenum, colon and liver of one irinotecan-treated animal

**Deleted:** (B) Immunoblot for an exemplary colon sample of one irinotecan-treated animal.

**Deleted:** (B) Immunoblot for an exemplary colon sample of one irinotecan-treated animal. ¶

**Figure 2:** The ICE Bioassay was used for the detection of topoisomerase I/DNA intermediates in stomach, small intestine, colon and liver of Wistar rats. ¶

In the pilot study (A + B), rats were treated with three doses of irinotecan hydrochloride x 3 H<sub>2</sub>O (50 mg/kg body weight). In the main study (C + D), rats received a single dose of irinotecan hydrochloride x 3H<sub>2</sub>O (100 mg/kg body weight).

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**Deleted:** Level of topoisomerase I/DNA intermediates detected in the pilot study (A) or in the main study (C), respectively. The data presented are the mean  $\pm$  SEM (pilot study: n =6 animal / group; main study: n=9 animals / group) relating to the DNA content. The indicated *p*-values (Student's *t* test) were referred to the respective control group. (B +D) Immunoblot of representative experiments: a, control group; b, irinotecan group. ¶

**Table 1:** Body (g) and organ weights (g) of control and irinotecan-treated groups. (mean  $\pm$  SEM);<sup>a)</sup>bw, body weight

| pilot study      |  | <sup>a)</sup> bw | stomach         | duodenum        | colon           | liver            |
|------------------|--|------------------|-----------------|-----------------|-----------------|------------------|
| (n = 6)          |  |                  |                 |                 |                 |                  |
| control group    |  | 293 $\pm$ 52     | 1.81 $\pm$ 0.37 | 1.00 $\pm$ 0.15 | 0.96 $\pm$ 0.42 | 12.75 $\pm$ 0.48 |
| irinotecan group |  | 249 $\pm$ 40     | 1.70 $\pm$ 0.27 | 0.81 $\pm$ 0.12 | 1.01 $\pm$ 0.29 | 12.54 $\pm$ 0.28 |
| main study       |  | <sup>a)</sup> bw | stomach         | duodenum        | colon           | liver            |
| (n = 6)          |  |                  |                 |                 |                 |                  |
| control group    |  | 266 $\pm$ 10     | 1.67 $\pm$ 0.07 | 1.23 $\pm$ 0.04 | 0.74 $\pm$ 0.08 | 11.60 $\pm$ 1.00 |
| irinotecan group |  | 263 $\pm$ 10.0   | 1.66 $\pm$ 0.07 | 1.18 $\pm$ 0.04 | 0.69 $\pm$ 0.07 | 12.60 $\pm$ 0.53 |

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. Furthermore also the organ weights (stomach, duodenum, colon and liver) did not differ significantly between groups (Table 1).

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The level of covalent DNA / topoisomerase I complexes in several organs of Wistar rats was determined using the ICE bioassay according to a modified method of Subramanian et al. [14]. Cell lysates were prepared from stomach, duodenum, colon and liver of rats

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treated intraperitoneally with a single dose of the topoisomerase I poison irinotecan hydrochloride x 3H<sub>2</sub>O (100 mg/kg body weight) or with 0.9% sodium chloride.

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cesium chloride gradient centrifugation of the lysate prepared from the stomach, the duodenum or the colon separated

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(data not shown). These data demonstrate that the used antibody is specific for

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irinotecan affects DNA integrity as a specific topoisomerase I inhibitor in rats

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Among the four selected organs

Figure 1

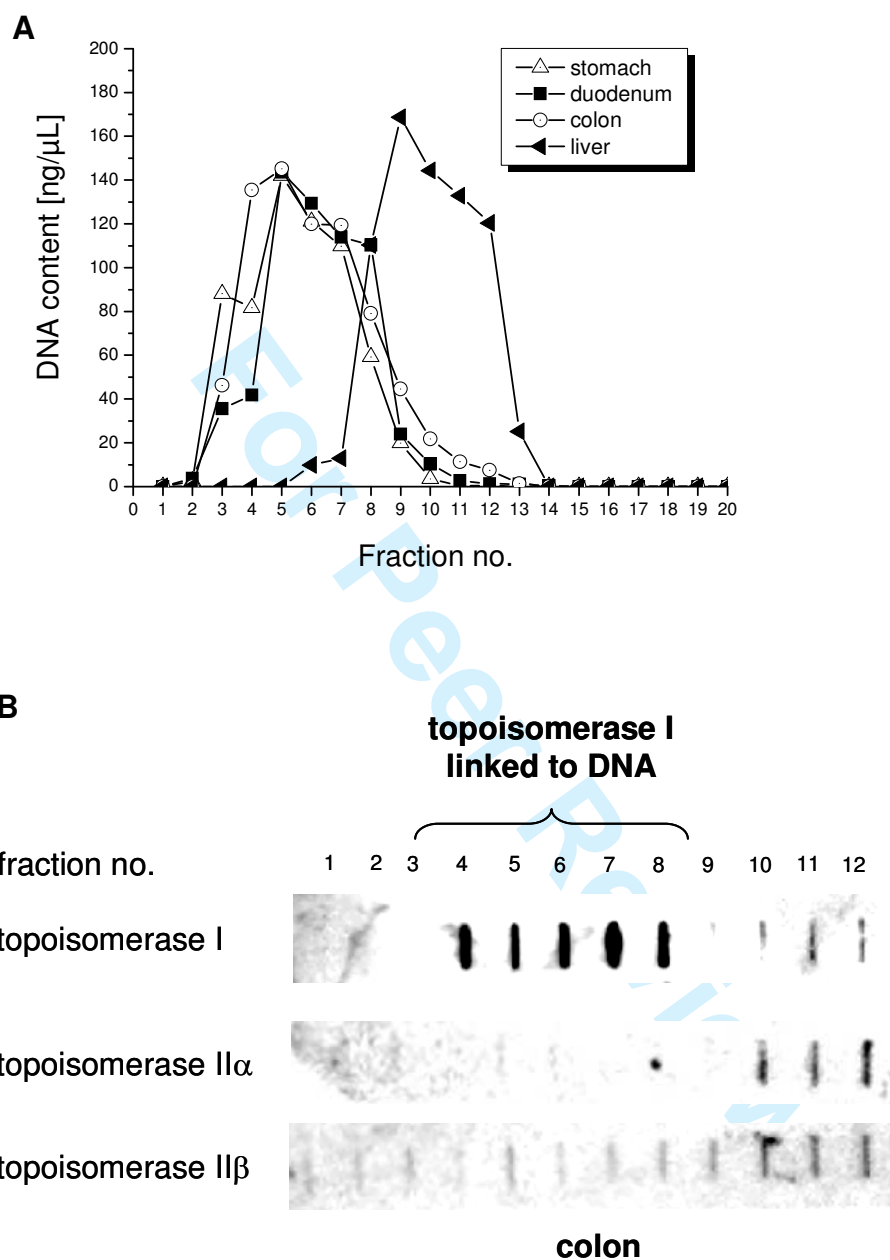
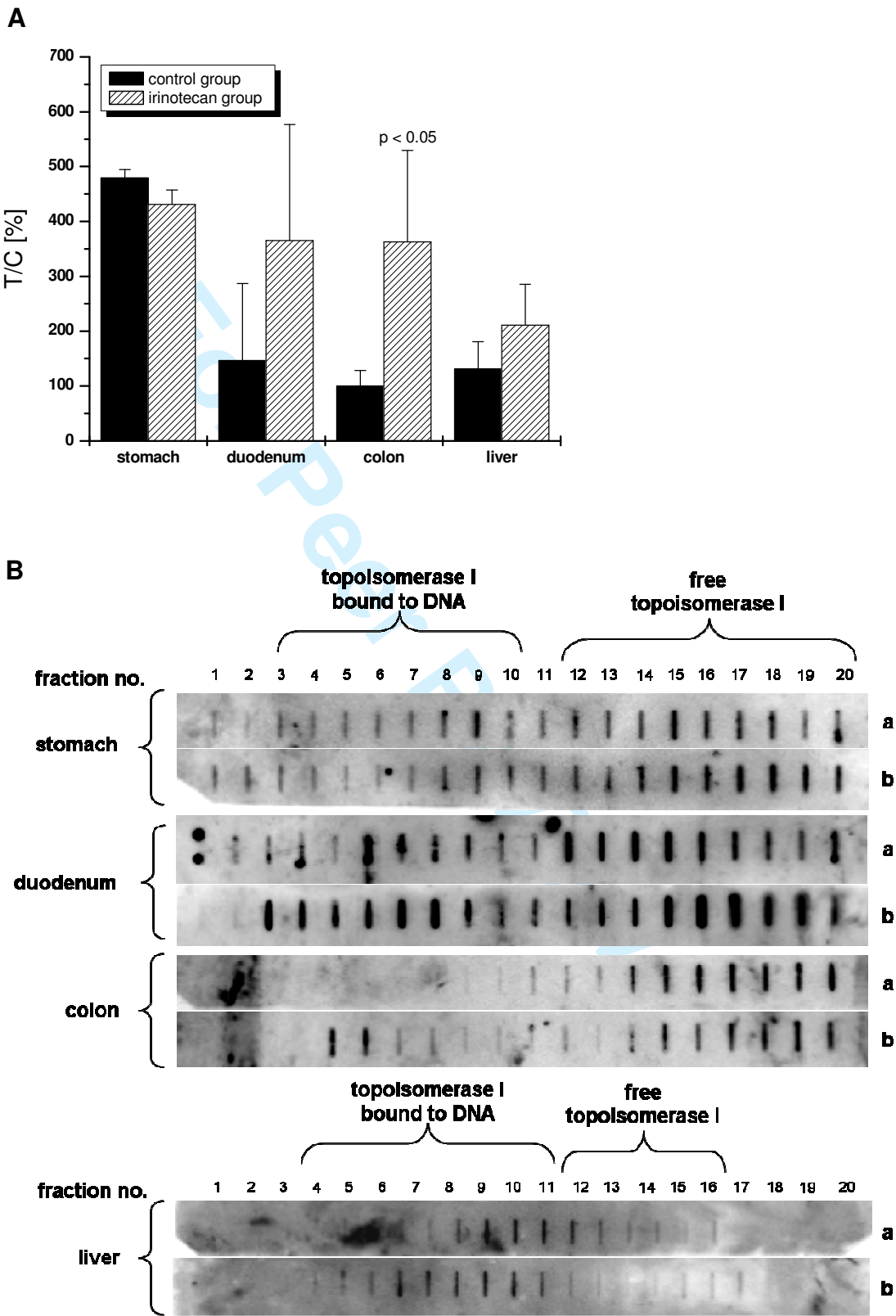
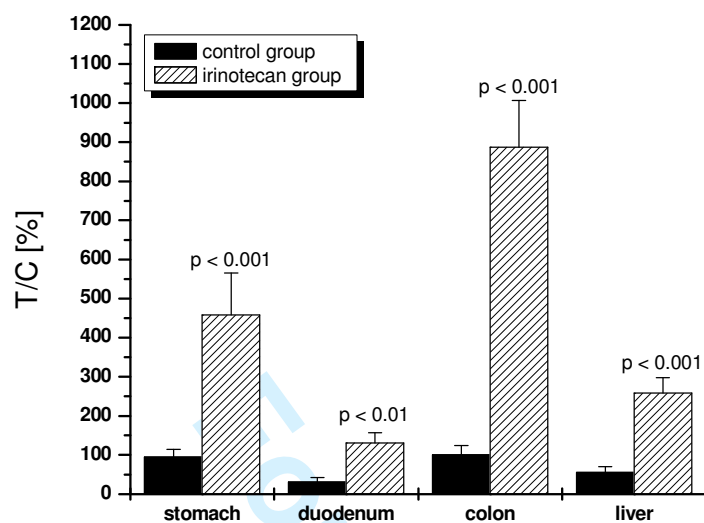


Figure 2



C



D

