Investigation of Bioequivalence Between Brand-name and Generic Irinotecan Products

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Abstract. *Background/Aim: To investigate bioequivalence among generic and brand-name irinotecan products. Materials and Methods: Products of Yakult and Daiichi-Sankyo (brand-name products), Sandoz, Nippon Kayaku, Taiho, and Sawai were compared with respect to their composition and antitumor activity. Results: High-performance liquid chromatography demonstrated that related substances were within the acceptable range. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay revealed significant differences in cytotoxicity for four cancer cell lines among the products. The concentration of the active compound SN-38 was highest in Yakult's product (23.82 ng/ml) and lowest in Daiichi-Sankyo's product (8.96 ng/ml). MTT assay data were correlated with the SN-38 concentration, suggesting that it influenced differences in cytocidal activity among products. However, the SN-38 concentration was far lower than that of irinotecan (20 mg/ml), suggesting a negligible clinical effect. Metabolism of irinotecan to SN-38 or open-ring forms did not differ significantly among the products. Conclusion: The generic products showed equivalent efficacy and safety to the brand-name products.*

Generic products are expected to show equivalence to brand-name products with respect to the dosage form, safety, efficacy and quality, while reducing medical expenses due to

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their lower cost. For oral drugs, equivalence between generic and brand-name products must be demonstrated by dissolution and bioequivalence tests. On the other hand, while quality assurance tests, such as purity tests, are required for injectable products, bioequivalence tests are not compulsory. Therefore, only certain companies voluntarily perform bioequivalence tests of injectable products. For anticancer agents, excluding some hormonal agents, bioequivalence studies in healthy volunteers are prohibited. However, some reports have been published comparing safety and pharmacokinetics between brand-name and generic injectable products. For example, the safety and pharmacokinetics of paclitaxel were reported to be similar between brand-name and generic products in patients with cancer (1-4). In contrast, it has been reported that generic forms of docetaxel, another taxane anticancer agent, cause more serious febrile neutropenia than the brand-name product (5). Moreover, cisplatin generics were found to cause more severe nephropathy and hematological toxicity than the brand-name product (6-8).

Irinotecan is a DNA topoisomerase I inhibitor derived from camptothecin that shows broad-spectrum strong antitumor activity (9). It is a prodrug, and carboxylesterases in the liver and other tissues convert it to the active metabolite, 7-ethyl-10-hydroxy-camptothecin (SN-38) (10), which has 1000-fold stronger pharmacological activity than the parent compound (11). Irinotecan is a key drug for treating various cancer types, including colorectal (12), lung (13), gastric cancer (14), breast (15), cervical (16), and ovarian (17). Generic products for irinotecan were released in Japan in May 2009.

Irinotecan is a semisynthetic derivative of camptothecin extracted from *Camptotheca acuminata* or *Nothapodytes foetida*, which are native to China (18, 19). It has been

Table I. *List of irinotecan products and manufacturers.*

Type of product	Name	Manufacturer
Brand name	Campto for *i.v.* infusion	Yakult Honsha
Generic	Irinotecan Hydrochloride Intravenous Infusion [SANDOZ]	Sandoz
Generic	Irinotecan for *i.v.* infusion [NK]	Nippon Kayaku Co., Ltd
Generic	Irinotecan Hydrochloride *i.v.* infusion 「TAIHO」	Taiho Pharmaceutical Co., Ltd.
Generic	Irinotecan Hydrochloride	Sawai Pharmaceutical Co., Ltd.
Brand name	Topotecin Intravenous Drip Infusion	Daiichi-Sankyo Co., Ltd.

reported that the plant used as a source material differs among pharmaceutical companies and that levels of contaminants vary as a consequence (20). Antitumor activity is also influenced by pH-related lactone ring opening, since the lactone form shows higher antitumor activity than the carboxylate form. These features of irinotecan suggest the potential for safety and pharmacokinetics to differ between the brand-name and generic products, but there have been no reports about this issue.

Accordingly, the present study was performed to investigate *in vitro* bioequivalence among the irinotecan products of various pharmaceutical companies by performing various tests, including assessment of purity, cytocidal activity, pH-dependent lactone ring opening, cytotoxicity of Y3 (a related substance), and formation of active metabolites by human hepatic microsomes. Our findings suggest that the brand-name and generic products would be expected to have equivalent efficacy and safety.

Materials and Methods

Anticancer agents. The reference formulation (brand-name product) was Campto® (Yakult Honsha,Tokyo, Japan), while the other products studied were Topotecin® (brand-name product) and four generic products (Table I). Authentic specimens of the following potential contaminants were supplied by Yakult Honsha: 3,10-diethyl-8-[(4-piperidinopiperidino)carbonyloxy]furo [3',4':6,7] indolizino-[1,2-b]quinoline-1,13(3*H*,11*H*)-dione (D1); 10-ethyl-2-methyl-3-propionyl-8-[(4-piperidinopiperidino)carbonyloxy] indolizino[1,2-b]-quinoline-1(11*H*)-one (D3); (4*S*)-4,11-diethyl-4,12-dihydroxy-9-[(4-piperidinopiperidino)carbonyloxy-1*H*-pyrano[3',4':6,7]-indolizino[1,2-b]quinoline-3,14(4*H*,12*H*)-dione (Y1); (4*S*)-4,11-diethyl-4,9,12-trihydroxy-1*H*-pyrano[3',4':6,7] indolizino[1,2-b]quinoline-3,14(4*H*,12*H*)-dione (Y3), and 4-ethyl-2-[6-(4-ethyl-4-hydroxy-3,8(1*H*,7*H*)-dioxopyrano[3,4-c]pyridyl)]-6-(piperidinopiperidino)-carbonyloxyquinoline-3-carboxylic acid (U1) (Figure 1).

Cell lines and culture. A small cell lung cancer cell line (PC-6) was obtained from the Second Department of Internal Medicine of Nagasaki University (Nagasaki, Japan), a non-small cell lung cancer cell line (PC-9) was from Kinki University (Higasiosaka-shi, Japan), and another non-small cell lung cancer cell line (A549) and an ovarian cancer cell line (NIH:OVCAR-3) were from the Riken Cell Bank, Tsukuba, Ibaraki, Japan.

A549 cells were incubated in Dulbecco's modified Eagle's medium (DMEM) (Wako, Osaka, Japan), while PC-6, PC-9, and NIH:OVCAR-3 cells were incubated in RPMI-1640 medium (Sigma-Aldrich, Tokyo, Japan). Both media contained 10% fetal bovine serum and incubation was at 37°C under 5% CO_2.

Assessment of purity. High-performance liquid chromatography (HPLC) was performed using a Hitachi Detector L-2400 and a Hitachi Pump L-2130 (Hitachi, Tokyo, Japan). The detection wavelength was 254 nm. The analytical column was a Capcell Pak C18 MG (5 μ, 3.0 Φ×150 mm) from Shiseido (Tokyo, Japan). Mobile phase A was a mixture of 50 mM formic acid buffer (pH 5.1), acetonitrile and MeOH at 75:10:15 (v/v/v), while mobile phase B was a mixture of 50 mM formic acid buffer (pH 5.1), acetonitrile and MeOH at 55:30:15 (v/v/v). Elution was performed at a flow rate of 1.0 ml/min and a temperature of 50°C using the following protocol: gradient elution from A to B (30 min)→A (1 min)→ equilibration (5 min).

Cytotoxicity test. Small cell lung cancer cells (PC-6), non-small cell lung cancer cells (PC-9, A549) and ovarian cancer cells (NIH:OVCAR-3) were used to evaluate cytotoxicity by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells in the logarithmic growth phase were suspended in 0.05% trypsin-EDTA (Nacalai Tesque, Kyoto, Japan) and adjusted to 5.6×10^3 cells/ml in irinotecan-free medium. This suspension was inoculated at a volume of 1,800 μl/well (1,000 cells/well) into a 96-well microplate, and serial dilutions of each product in medium were added to the wells. After 72 h of incubation, 5 mg/ml MTT (Nacalai Tesque) was added at 20 μl/well and incubation was continued for 4 h. Then centrifugation (400 × *g* at 4°C) was performed for 10 min. After the medium was discarded, dimethyl sulfoxide was added at a volume of 200 μg/well, and the absorbance was measured at 570 nm using a microplate reader (reference wavelength of 650 nm).

Formation of open-ring irinotecan. Irinotecan adopts closed- and open-ring forms under acidic and basic conditions, respectively (21). Because opening the lactone ring alters the antitumor activity of irinotecan, the extent of formation of its open-ring form was compared among the products. During incubation of each product in human plasma (pH 7.4±0.05) at 37°C, samples were collected over time and deproteinized for analysis by HPLC (fluorescence detector: excitation at 380 nm and emission at 550 nm). The concentration of the closed-ring form relative to the total irinotecan
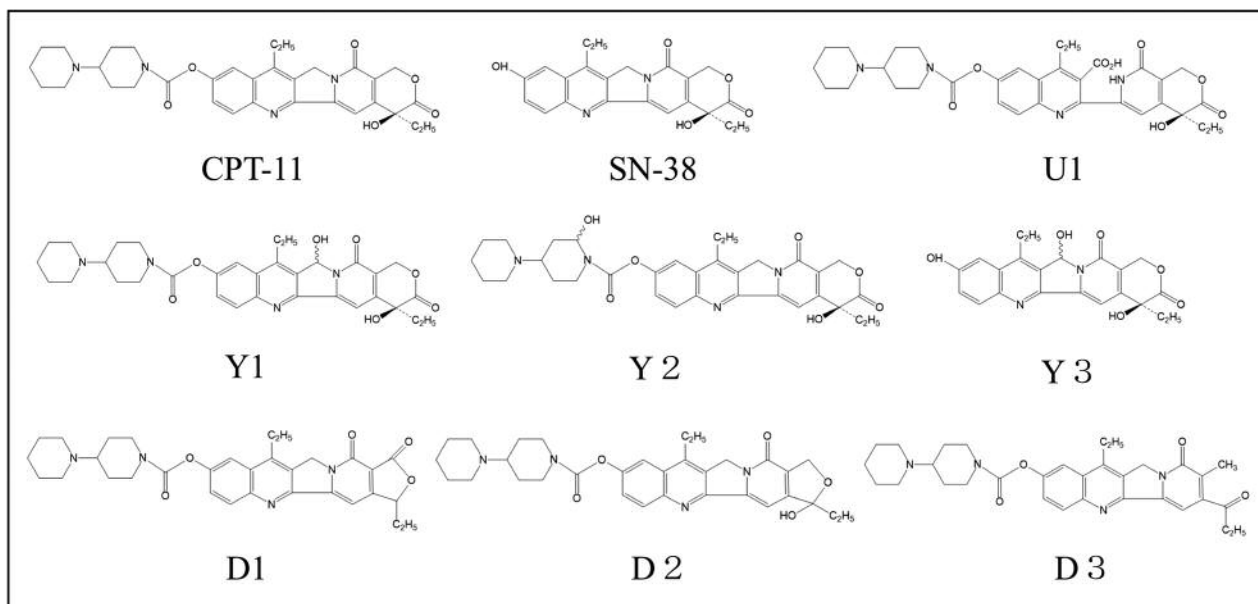
Figure 1. *Structure of irinotecan and related substances.*

concentration was determined over time, with the concentration of the closed-ring form at the start of incubation being set at 100%.

SN-38 formation by human hepatic microsomes. An aliquot (30 μl) of 25 mg/ml human hepatic microsomes (Biopredic, Saint-Grégoire, France) was solubilized by adding 6 μl of 1% Triton X-100. The solubilized microsomes were added to 60 μl of NaH_2PO_4 (0.1 mol/l pH 7.4) together with 51 μl of water and 3 μl of irinotecan (0.8 mg/ml) for incubation at 37°C. Samples were collected after 2, 4, and 6 min and were immediately added to acetonitrile on ice (0°C) for deproteinization. After centrifugation for 5 min at $21,000 \times g$ and 4°C, 10 μl of the supernatant was injected into the HPLC system by an autosampler for analysis (fluorescence detector: excitation at 380 nm and emission at 550 nm).

Cytotoxicity of Y3 and SN-38. Because the potential contaminant Y3 has a similar structure to SN-38, even a low content of Y3 can influence a product's cytocidal activity. Therefore, the cytotoxicity of Y3 for small-cell lung cancer cell line PC-6 and non-small cell lung cancer cell line PC-9 was evaluated by the MTT assay and the Y3 content of each product was measured by HPLC.

Results

Comparison of impurities among the products. When impurities were measured by HPLC and compared among the products, peaks of related substances with known structures (such as D1, D3, Y1, and U1) were detected in addition to the peaks of irinotecan and SN-38 (Table II). While the peak area of each related substance varied among the products, it was always less than 0.2%, which is the threshold specified by "Impurities in New Drug Products" in

the International Conference on Harmonisation (ICH) guidelines (22). Peaks of unknown contaminants were also noted, but the peak area of each contaminant was less than 0.1%. These results suggest that the products were equivalent with respect to their impurities.

Cytotoxicity. When *in vitro* cytocidal activity was compared between the products by the MTT assay (Table III), the 50% inhibitory concentration (IC_{50}) for PC-6 showed a significant difference between the reference formulation and three other products.In addition, the IC_{50} values for PC9 and NIH:OVCAR-3 cells were significantly different between the reference formulation and four or five other products, respectively. When the SN-38 content was measured in the impurity test, it was significantly lower in the products of Sandoz, Sawai, and Daiichi-Sankyo than in the reference formulation (Table IV). The difference of IC_{50} against PC-6 cells among the products was significantly related to the SN-38 content ($r^2 = 0.813$, $p < 0.05$) as it was against PC-9 cells ($r^2 = 0.951$, $p < 0.01$), but not A549 cells ($r^2 = 0.326$, $p > 0.05$) nor NIH:OVCA R-3 cells ($r^2 = 0.123$, $p > 0.05$). Yakult's product had the highest SN-38 content (23.82 ± 3.55 ng/ml) and the strongest cytotoxicity, while the product of Daiichi-Sankyo had the lowest SN-38 content (8.96 ± 0.62 ng/ml) and tended to exhibit weaker cytotoxicity than the other products against all cell lines.

Open-ring form. When each product was incubated in human plasma, the decrease in the lactone (closed-ring) form of irinotecan over time did not significantly differ among the

Table II. *Peak area ratios of irinotecan and contaminants. High-performance liquid chromatography peak area (%) profiles of the reference formulation and other products are shown. The peak area ratio of each contaminant was <0.2% and was acceptable by International Conference on Harmonisation guidelines.*

	Product manufacturer					
Compound	Yakult	Sandoz	Nippon Kayaku	Taiho	Sawai	Daiichi-Sankyo
Irinotecan (closed)	99.56	99.52	99.60	99.46	99.52	99.47
Irinotecan (open)	0.14	0.18	0.20	0.22	0.11	0.14
D1	0.05	0.01	0.02	0.01	0.01	0.01
D3	0.10	0.08	0.06	0.11	0.09	0.11
Y1	0.04	0.06	0.01	0.06	0.08	0.06
U1	0.06	0.02	0.06	0.02	0.05	0.07
Unknown 1	n.d.	n.d.	0.01	0.01	0.01	0.04
Unknown 2	n.d.	0.06	0.01	0.04	0.05	0.01
Other	0.05	0.07	0.03	0.07	0.08	0.09
Total	100.00	100.00	100.00	100.00	100.00	100.00

Table III. *Cytotoxicity of each product. The 50% inhibitory concentration (IC_{50}) of each product was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The cytotoxicity of the other products was weaker than that of the Yakult reference formulation.*

	IC_{50} (μM)					
	Product manufacturer					
Cell line	Yakult	Sandoz	Nippon Kayaku	Taiho	Sawai	Daiichi-Sankyo
PC-6	1.37±0.05	1.86±0.06**	1.38±0.08	1.59±0.09**	1.39±0.05	2.03±0.07**
PC-9	7.46±0.68	12.36±0.47**	8.01±0.51	9.07±0.48**	9.14±0.29**	16.70±0.56**
A549	44.99±6.90	77.09±8.89**	64.07±10.21**	75.17±7.95**	61.61±7.63**	72.32±6.89**
NIH:OVCAR-3	16.66±0.71	20.85±1.37**	23.08±3.44**	24.70±2.26**	24.63±0.95**	24.08±2.13**

Data are the mean±SD. **Significantly different at $p<0.005$ *vs.* Yakult by Student's *t*-test.

products (Figure 2), and the formation rate of the open-ring form was considered to be equivalent among the products.

Metabolism to SN-38. When the conversion rate of irinotecan to SN-38 by human hepatic microsomes was investigated, there were no significant differences among the products (Table V).

Cytotoxicity of Y3 and SN-38. The IC_{50} of Y3 for PC-6 and PC-9 cells was 24-fold and 64-fold higher than that of SN-38, respectively (Table VI). The Y3 content of each product was similar to or significantly lower than that of the reference formulation (Table IV). In addition, the Y3 content was approximately 45, 20, 11, 8, 23 and 109 times higher than the SN-38 content in the products of Yakult, Sandoz, Nippon Kayaku, Taiho, Sawai, and Daiichi-Sankyo, respectively (Table IV). Even though Y3 was less toxic than SN-38, these differences in Y3 content might have influenced the cytotoxicity of the products. In fact, Yakult's
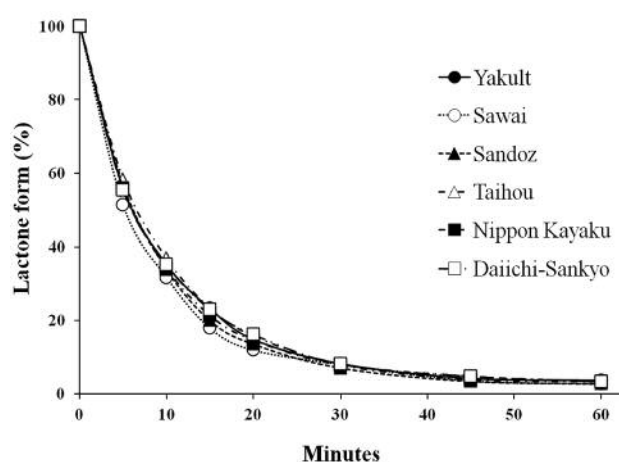

Figure 2. *Elimination rate of the lactone form of irinotecan. Each irinotecan product was incubated in human blood plasma in vitro. The lactone form was changed to carboxylate form with increasing pH over time. There were no significant differences between products at each time point.*

Table IV. *SN-38 and Y3 concentration in each product. High-performance liquid chromatography showed that the reference formulation had the highest concentration of SN-38.*

Compound	Product manufacturer					
	Yakult	Sandoz	Nippon Kayaku	Taiho	Sawai	Daiichi-Sankyo
SN-38 (ng/ml)	23.82±3.55	14.59±2.14**	22.91±4.20	21.56±0.57	18.16±0.86*	8.96±0.62**
Y3 (ng/mI)	1069.7±64.7	293.3±91.1**	243.8±74.3**	168.4±45.5**	413.3±67.1**	974.0±166.9

Data are the mean±SD. Significantly different at *$p<0.05$ and **$p<0.005$ *vs*. Yakult by Student's *t*-test.

Table V. *Formation of SN-38 by human hepatic microsomes. There were no appreciable differences in metabolism to SN-38 among the products by Student's t-test.*

	Product manufacturer					
	Yakult	Sandoz	Nippon Kayaku	Taiho	Sawai	Daiichi-Sankyo
ng/min/mgP	1.21±0.14	1.15±0.07	1.33±0.04	1.38±0.06	1.40±0.14	1.40±0.07

Data are the mean±SD.

product, with the highest content of SN-38 and Y3, had the strongest cytotoxicity (Table III).

Discussion

In this study, we evaluated the *in vitro* bioequivalence of various irinotecan products and we clarified the following points. Firstly, levels of contaminants differed among the products, but were always within the acceptable range specified by the guidelines. Secondly, cytotoxicity differed significantly among the products, and these differences probably reflected differences of the SN-38 content. Finally, there were no significant differences of pH-dependent ring-opening or metabolism to SN-38. Because irinotecan is a semisynthetic derivative of camptothecin, each product was subjected to HPLC to determine the levels of active ingredients and contaminants, and we also compared cytotoxicity, metabolism, and pH-dependent ring opening.

Various contaminants (D1, D3, Y1, U1, *etc*.) were detected in addition to irinotecan and its active metabolite SN-38. The content of each related substance differed among the products, presumably due to differences in the raw materials and manufacturing methods, but this was considered to be of no clinical relevance because the content of each substance was always within the acceptable range according to the ICH guidelines. Comparison of pH-dependent irinotecan ring opening also did not significantly differ among the products. However, the MTT assay revealed significant differences of cytotoxicity among the products. Formulations of irinotecan contain trace levels of various decomposition products and contaminants,

Table VI. *Cytotoxicity of Y3 and SN-38. The 50% inhibitory concentration (IC_{50}) values of Y3 and SN-38 were measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay using cancer cell lines (PC-6 and PC-9). The cytotoxicity of Y3 was much weaker than that of SN-38.*

Compound	Mean IC_{50}±S.E. (nM) Cell line	
	PC-6	PC-9
SN-38	39.7±3.2	45.2±3.3
Y3	933.5±40.2	2898.4±154.8

including the active metabolite SN-38, with far stronger antitumor activity than irinotecan (100 to several thousand times higher) (23, 24). This study showed that the SN-38 concentration of each product (8.96-23.82 ng/ml) was far lower than the irinotecan concentration (20 mg/ml). If irinotecan was partially metabolized to SN-38 during the MTT assay, the amount of SN-38 produced would be far higher than the initial content in each product, which might suggest there was little likelihood of the differences in the baseline concentration of SN-38 causing the differences in cytotoxicity among the products. However, there was no significant difference in the metabolism of irinotecan to SN-38 by hepatic microsomes among the products and cytotoxicity in the MTT assay was related to the concentration of SN-38 in each product, suggesting that the differences in cytotoxicity among the products were actually related to differences of the baseline SN-38 concentration.

We also compared cytotoxicity between SN-38 and Y3, a related substance with a similar structure to SN-38. Although the cytotoxicity of Y3 was lower than that of SN-38, the Y3 content of each product was 8-109 times higher, suggesting that Y3 was likely to influence the results of the MTT assay. In fact, Yakult's product, with the highest SN-38 and Y3 content, had the strongest cytotoxicity and the product of Daiichi-Sankyo with a Y3 content 109-fold higher than that of SN-38 (8.96 ± 0.62 ng/ml vs. 974.0 ± 166.9 ng/ml) exhibited relatively strong cytotoxicity against A549 and NIH:OVCAR-3 cells.

However, it must also be considered whether such $in vitro$ differences among the products could lead to clinical differences in safety and pharmacokinetics. The concentration of irinotecan in each product was 20 mg/ml, which was many times higher than that of SN-38 (8.96-23.82 ng/ml) and Y3 (168.4-1,069.7 ng/ml). Moreover, the area under the plasma concentration–time curve for plasma SN-38 was found to be approximately 0.03-0.08 times that of irinotecan in clinical studies (25-27). The SN-38 concentrations of the products were much lower than that generated from irinotecan in plasma. Hence, the differences of SN-38 concentrations of the products would have little influence on $in vivo$ cytotoxicity, indicating that each product is considered to be equivalent. The SN-38 concentrations of the generic products were similar to or lower than that of reference formulation, indicating that there is little likelihood of the generic products having stronger clinical cytotoxicity.

In summary, based on the results of the present study, we concluded that each irinotecan product tested was equivalent to the reference formulation.

Irinotecan causes various adverse reactions, including myelosuppression and diarrhea (28, 29). We consider that bioequivalence should be investigated for injectable drugs with strong toxicities such as anticancer agents. If possible, clinical studies should be performed to assess the safety and efficacy of generic products before their approval. Although large-scale clinical studies are undoubtedly expensive, $in vitro$ studies such as the present investigation can provide an indication of bioequivalence. In the future, it would seem necessary to conduct further studies to determine whether the differences among products that we identified influence the efficacy and adverse reactions of these products in clinical practice.

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