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In Vitro Pharmacodynamics of CKD-602 in HT-29 Cells

In-Sook Park, Mee Ryung Ahn, Soo Kyung Suh, Hong-Serck Choi, Soo Jung Sohn, Ji Sun Yang, Tae Moo Yoo[2], and Hyo-Jeong Kuh[1,2]

*Department of Pharmacology, National Institute of Toxicological Research, Korea Food and Drug Administration, 5 Nokbun-Dong, Eunpyung-ku, and [1]Catholic Research Institutes of Medical Science, The Catholic University of Korea, 505 Banpo-dong, Seocho-ku, Seoul, Korea. [2]**Corresponding authorship** is shared by Hyo-Jeong Kuh and Tae Moo Yoo*

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CKD-602 (7-[2-(N-isopropylamino)ethyl]-(20S)-camptothecin) is a recently-developed synthetic camptothecin analogue and currently under clinical development by Chong Kun Dang Pharm (Seoul, Korea). CKD-602 showed potent topoisomerase inhibitory activity *in vitro* and broad antitumor activity against various human tumor cells *in vitro* and *in vivo* in animal models. This study describes the pharmacodynamics of the immediate and delayed cytotoxicity induced by CKD-602 in a human colorectal adenocarcinoma cell line, HT-29, and its intracellular drug accumulation by HPLC. The present study was designed to address whether the higher activity of CKD-602 with prolonged exposure is due to delayed exhibition of cytotoxicity and/or an accumulation of antiproliferative effect on continuous drug exposure. The drug uptake study was performed to determine whether the delayed cytotoxicity is due to a slow drug accumulation in cells. CKD-602 produced a cytotoxicity that was exhibited immediately after treatment (immediate effect) and after treatment had been terminated (delayed effect). Both the immediate and delayed effects of CKD-602 showed a time dependent decrease in IC_{50} values. Drug uptake was biphasic and the second equilibrium level was obtained as early as at 24hr, indicating that the cumulative and delayed antitumor effects of CKD-602 were not due to slow drug uptake. On the other hand, CKD-602 treatment was sufficient to induce delayed cytotoxicity after 4hr, however, longer treatment (>24hr) enhanced its cytotoxicity due to the intracellular accumulation of the drug, which requires 24hr to reach maximum equilibrium concentration. In addition, $C^n \times T=h$ analysis (n=0.481) indicated that increased exposure times may contribute more to the overall antitumor activity of CKD-602 than drug concentration. Additional studies to determine the details of the intracellular uptake kinetics (e.g., concentration dependency and retention studies) are needed in order to identify the optimal treatment schedules for the successful clinical development of CKD-602.

Key words: CKD-602, Intracellular uptake, Immediate effect, Delayed effect. Pharmacodynamics

INTRODUCTION

Camptothecin is a plant antitumor alkaloid isolated from *Camptotheca acuminata* (Wall *et al.*, 1966). Camptothecin analogues constitute a new class of anticancer agents targeting a nuclear enzyme, topoisomerase I (Kohn and Pommier, 2000; Creemers *et al.*, 1994; Slichenmyer *et al.*, 1993). In the United States, two camptothecins, irinotecan (CPT-11) and topotecan have been approved by the FDA for the treatment of previously treated metastatic colorectal cancers and for advanced refractory ovarian cancers, respectively (Takimoto *et al.*, 1998). Studies upon the clinical development of camptothecins have focused on useful combination regimens with other active agents, and the establishment of the best schedules for single and combination therapies (Meibohm and Derendorf 1997; Gerrits *et al.*, 1999; Jung and Zamboni 2001; Li *et al.*, 2001).

CKD-602, 7-[2-(*N*-isopropylamino)ethyl]-(20*S*)-camptothecin, is a synthetic water soluble camptothecin analogue, which is being developed by Chong Kun Dang Pharm., Seoul, Korea (Jew *et al.*, 1998). CKD-602 showed broad

Correspondence to: Hyo-Jeong Kuh, Ph.D. Catholic Research Institutes of Medical Science Catholic University of Korea 505 Banpo-dong, Seocho-ku Seoul 137-701 Korea
E-mail: hkuh@cmc.cuk.ac.kr

antitumor activity against various human tumor cells with potency equivalent to or greater than those of camptothecin and topotecan (Lee *et al.*, 1998). CKD-602 was shown to have 4 times the potency of topotecan in the inhibition of topoisomerase activity *in vitro*. Its *in vivo* antitumor activity has also been demonstrated against L1210 leukemia models and the maximum tolerated dose (MTD) of CKD-602 was found to be 25 mg/kg on a Q4dx4 schedule in mice bearing L1210 leukemia (Lee *et al.*, 1998).

A phase I study of CKD-602 was performed using a 30min infusion on a 5-consecutive day every 3 wk schedule (Lee *et al.*, 2000). MTD was determined as 0.7 mg/m^2/day and dose-limiting toxicity (DLT) was neutropenia. Partial responses were observed in patients with stomach and ovarian cancers, and subsequently Phase II clinical trials of CKD-602 are under way. As for other camptothecin analogues, further studies upon the development of CKD-602 include the identification of other active agents for useful combination regimens and the establishment of the best schedule for single and combination administrations (Jung and Zamboni 2001; Li *et al.*, 2001).

In the preliminary study, longer exposure times resulted in the higher activity of CKD-602. Hence, we evaluated its immediate and delayed cytotoxicity *in vitro*, to determine whether this higher activity upon prolonged exposure to CKD-602 was due to delayed exhibition of cytotoxicity and/or the accumulation of an antiproliferative effect requiring continuous drug exposure in HT-29 human colorectal cancer cells (Au *et al.*, 1998). We also studied the concentration- and time-dependency of the cytotoxic effect of CKD-602 and its intracellular drug accumulation kinetics to determine whether its delayed cytotoxicity is due to a slow drug accumulation in cells. The relative importances of concentration and time for antitumor activity were analyzed using a $C^n \times T=h$ model, to provide useful information for designing *in vivo* studies as well as human clinical trials (Skipper 1965; Kalns *et al.*, 1995; Levasseur *et al.*, 1998).

MATERIALS AND METHODS

Chemicals

CKD-602 was kindly provided by Chong Kun Dang Pharm., (Seoul, Korea). RPMI1640, streptomycin/penicillin, trypsin/EDTA, and fetal bovine serum were purchased from GIBCO BRL (Gaithersburg, MD, USA). MTT, potassium phosphate monobasic, potassium phosphate dibasic, perchloric acid and triethylamine were purchased from Sigma Chemical Co. (St. Louis, MO., USA). PIC B-6 reagent was obtained from Waters Associates Inc., (Milford, MA, USA) and HPLC grade methanol and phosphoric acid(85%) from Fisher Scientific Co. (Fair Lawn, NJ., USA). The Cell Titer 96 Non-Radioactive Cell Proliferation Assay kit was purchased from Promega (Madison, WI, USA).

Cell lines and cell culture conditions

Human colorectal adenocarcinoma cell line, HT-29 was obtained from ATCC (American Type Culture Collection, Manassas, VA, USA) and maintained in RPMI1640 containing 10% fetal bovine serum, 100 IU/l penicillin, 10 μg/ml streptomycin in a CO_2 incubator (O_2(95%) and CO_2(5%)) at 37°C.

Cytotoxicity assay

Cytotoxic effect was determined using MTT assay. Cells in the log phase of growth were harvested and plated at 1×10^4 cells/well in 96 well plates. After 24 hr of preincubation, CKD-602 was added to the culture medium at final concentrations of 15.6, 31.25, 62.5, 125, 250, 500, 1000, and 2000nM. Cytotoxicity was measured after 6, 24, 48, and 72hr of incubation to determine immediate effects. For delayed effects, drug-containing medium was removed at predetermined times (4, 24, 48, 72, and 96hr) and cells were further incubated in drug free medium until 96hr when the cytotoxicity was finally determined. MTT assay was performed according to a general procedure. Briefly, the culture medium was removed at the end of the incubation period and 50 μl of MTT solution (1 mg/ml) was added. After 4 hr of incubation, the formazan crystals were dissolved by adding 150 μl of DMSO, and absorbance was measured at 570 nm.

Intracellular drug uptake study

Cells were plated at 2×10^5/well in a 24-well plate and preincubated for 24 hr before drug treatment. CKD-602 was added to the culture medium at 1000nM and the supernatant culture medium and cells were collected after 0, 10, 20, 30, 60, 120, and 240min, and 8, 12, 24, 48, and 72 hr. To determine cellular drug concentration, after removing the medium, the cells were briefly washed with ice cold PBS and harvested using trypsin and suspended in 0.3 ml of PBS. The cell suspension was stored at -70°C until required for HPLC analysis.

HPLC conditions for measurement of CKD-602 concentration.

The HPLC method described by Beijnen *et al.* was adopted (Oguma *et al.*, 2000; Beijnen *et al.*, 1990). The drug concentrations in the medium and cells were determined. Drug was extracted from medium by using methanol and 7% perchloric acid extraction. After vortexing for about 20sec, the mixture was centrifuged and 50 μl of the supernatant was injected into a HPLC.

Cell suspension was subjected to 3 repeated cycles of freeze-thaw and 20sec sonication before methanol/7% perchloric acid extraction. A C_{18} reversed phase column (Nucleosil C_{18}, 4 mm × 25 cm, particle size 5 μm) and a C_{18} guard column were used with a mobile phase, which contained 1M phosphate buffer (pH6.0), methanol, water, PIC B and triethylamine (50:860:600:25:5, v/v) with pH adjusted to 6 by 85% phosphoric acid. HPLC systems consisted of a pump (Younglin, M930D), automatic sample injector (TSP), and a fluorescence detector (Millipore). At a flow rate of 1.0 ml/min, the retention time of CKD-602 was 4.7 min. Excitation at 234nm and emission at 434 nm was used to detect CKD-602. Quantification was based on peak area and the calibration curve was linear in the range of 625nM to 10,000nM.

Data analysis

Cytotoxicity data were obtained as % control and analyzed using the E_{max} model (Eq. 1).

$$\%\text{Cell viability} = (100-R)\times\left(1-\frac{[D]^m}{K_d^m+[D]^m}\right)+R$$

where D is the drug concentration, K_d is the concentration of drug that produces a 50% reduction in absorbance (i.e., IC_{50}), *m* is the Hill-type coefficient, and R the residual unaffected fraction (resistance fraction). The relative significance of concentration and the time of exposure was determined using a $C^n \times T = h$ model (Levasseur *et al.*, 1998) and the Sigmaplot regression function was used for model fitting.

RESULTS AND DISCUSSION

In vitro cytotoxicity of CKD-602 in HT-29 cells

The *in vitro* cytotoxicity of CKD-602 in HT-29 cells was determined by using the MTT assay. The % cell viability was measured in two ways. The immediate effect was determined at the predetermined drug exposure times, and the delayed effect after incubating in drug-free media until 96 hr after removing drug-containing media at predetermined times. For the immediate effect, no decrease in cell viability was observed after 6hr of exposure over the tested concentration range of 62.5 nM to 2000 nM and a maximum of 66% growth inhibition was observed after 72hr exposure (Fig. 1). When exposed for 24 hr, concentrations under 500nM appeared to be nontoxic in HT-29 cells (Fig. 1). Immediate effect of CKD-602 showed a time dependent decrease in the resistance fraction (R) and the IC_{50} values (Table 1).

The delayed effect of CKD-602 showed pharmacodynamics that differed from the immediate effect (Fig. 2

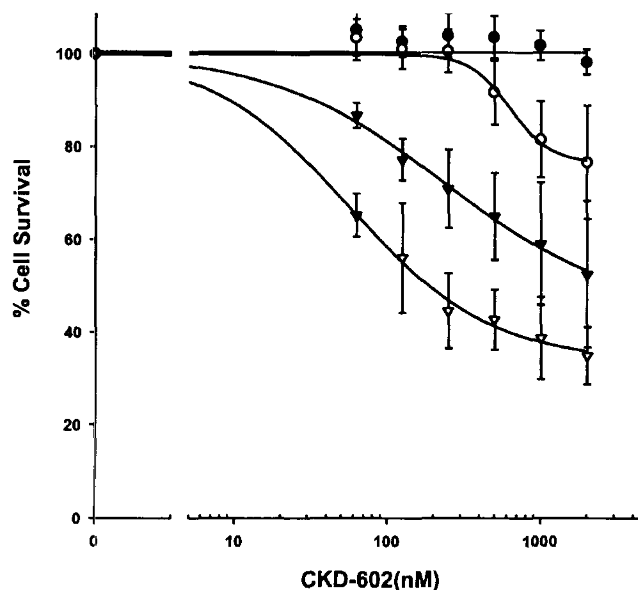

Fig 1. Immediate cytotoxicity of CKD-602 in HT-29 cells. Cells were treated with 62.5nM to 2000nM of CKD-602 for 6 (●), 24 (○), 48 (▼) and 72 (▽) hr and its cytotoxicity was measured immediately after exposure using MTT assay. Each data point represents the average ± SD of three experiments.

Table 1. Pharmacodynamic parameters of the immediate effect of CDK-602 in HT-29 cells. The immediate effect was determined right after the predetermined drug exposure times. Cytotoxicity data were obtained as % control absorbance and analyzed using an E_{max} model (Eq. 1, see Methods).

Exposure times	IC_{50} (nM)	R^a (%)	m^b
6 hr	ND^c	108.0	ND
24 hr	631.9	76.72	3.32
48 hr	236.7	44.42	0.769
72 hr	56.9	33.76	0.94

[a]R, the residual unaffected fraction (resistance fraction), see Eq. 1
[b]*m* is the Hill-type coefficient, see Eq. 1
[c]ND, not determined.

and Table 2). Although the E_{max} model estimated a lower R (4.91%) for 4hr exposure than those exposed for longer times, the apparent maximum growth inhibition was 83% (Fig. 2). IC_{50} drastically decreased from 1310 nM at 4hr to 39.32 nM at 96hr. With increasing exposure time from 24 hr to 96hr, the IC_{50} decreased by 70% while the maximum growth inhibition, i.e., (100−R), showed only a 10% increase (Table 2).

Comparison of the dose-response curves for the immediate and delayed effects showed that the delayed effect after 24 hr, 48 hr, and 72 hr was greater than the corresponding immediate effect, indicating that there was a delayed exhibition of cytotoxicity of CKD-602. On the other hand, both immediate and delayed effects up to 72hr exposure showed significant increases in cytotoxicity

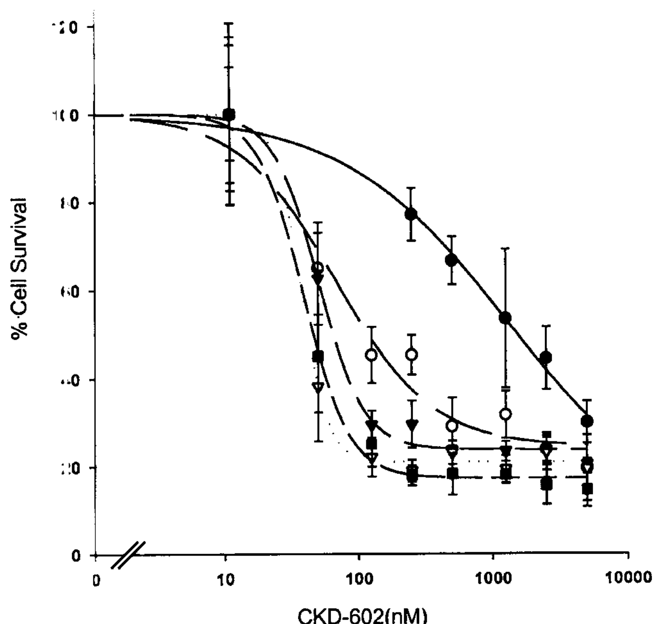

Fig 2. Delayed cytotoxicity of CKD-602 in HT-29 cells. Cells were treated with 7.8nM to 2000nM of CKD-602 for 4 (●), 24 (○), 48 (▼) 72 (▽) and 96 (■)hr. Drug-containing media was removed after each drug exposure and cells were incubated in drug-free media until 96 hr. Each data point represents the average ± SD of three experiments.

Table 2. Pharmacodynamic parameters of the delayed effect of CDK-602 in HT-29 cells. Delayed effect was determined after incubating in drug-free media until 96hr after removing the drug-containing media at predetermined times. Cytotoxicity data were obtained as % control absorbance and analyzed by E_{max} model (Eq. 1, see Methods).

Exposure times	IC_{50} (nM)	R^a (%)	m^b
94 h	1314	4.91	0.707
24 h	97.75	24.35	1.2
48 h	50.76	23.70	2.59
72 h	37.36	20.96	4.39
96 h	39.32	17.28	2.58

[a]R, the residual unaffected fraction (resistance fraction), see Eq. 1
[b]*m* is the Hill-type coefficient, see Eq. 1

with longer exposures, indicating that there was a cumulative cytotoxicity of CKD-602, as well.

Collectively, these results demonstrate that CKD-602 has a cytotoxicity that is exhibited immediately after treatment (immediate effect) and one after treatment is terminated (delayed effect), and that the maximum antiproliferative activity of CKD-602 in HT-29 cells, similar to 9-aminocamptothecin, may be obtained by increasing drug exposure time to 72 hr. However, exposures longer than 72 hr may not result in increased cytotoxicity (Jung and Zamboni 2001; Li *et al.*, 2001). Even with 96hr exposure, 20% of the cells appeared to be resistant to

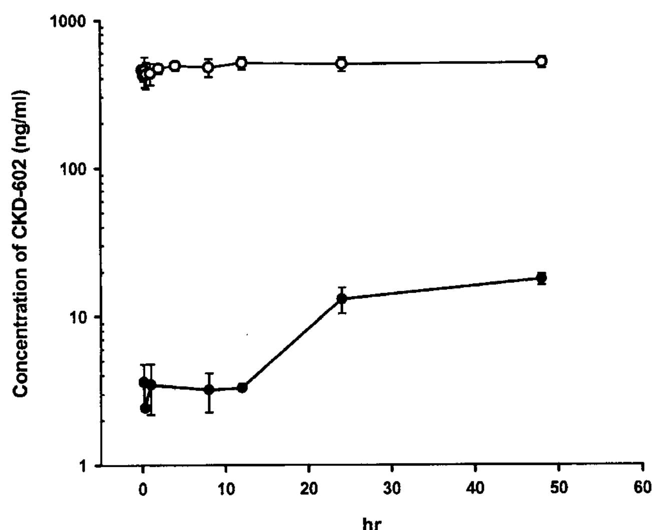

Fig 3. The concentration of CKD-602 in medium (○) and in cell suspension (●) when cells were exposed at 1000nM for 48hr. Drug concentration was measured by HPLC. Intracellular drug concentration was estimated as 1540 ng/ml at 1hr and 3530 ng/ml at 24 hr (see Results and Discussion for details). Each data point represents the average ± SD of three experiments.

CKD-602 (Fig.2, Table 2), suggesting that combination with other anticancer drugs may be necessary to improve response rate and achieve better efficacy.

Intracellular accumulation of CKD-602 in HT-29 cells

As mentioned above, comparisons of the dose-response curves of immediate and delayed effects indicated that there was delayed cytotoxicity of CKD-602 in HT-29 cells. Whether a slow cellular uptake of CKD-602 caused this delayed effect was determined by measuring the concentration of CKD-602 in the medium and cells over a 48 hr-incubation (Fig. 3). The intracellular uptake was found to be biphasic: the initial concentration was maintained at 3-4 ng/ml for 12 hr and the second equilibrium was obtained at 24 hr. The data in Fig 3 are expressed in ng/ml, and represent the amount of drug contained in a cell suspension prepared for cell harvest. Taking an average cell volume of 3.4×10^{-9} ml for one cell and a doubling time of 24 hr, the intracellular concentration was estimated to be 1540 ng/ml at 1hr and 3530 ng/ml at 24 hr. On comparing the with the intracellular and medium concentrations of CKD-602, its intracellular concentration was found to be 3.3 fold and 7.7 fold higher at 1 hr and 24 hr, respectively. The drug concentration in medium did not change over 48 hr, indicating no degradation of CKD-602 in the medium and that the fraction of drug taken up by the cells was insignificant (maximum drug uptake in cells was

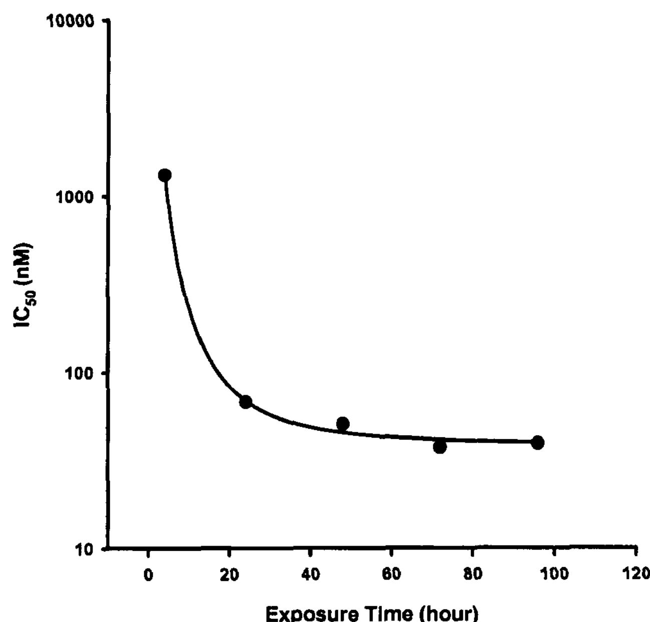

Fig 4. Model fitting of $C^n \times T=h$ for IC_{50} of CKD-602 in HT-29 cells. Each data point represents the average IC_{50} after 4, 24, 48, 72, and 96 hr of exposure. The relative significances of the concentration and the time of drug exposure were determined using the $C^n \times T=h$ model (Levasseur *et al.*, 1998). The Sigmaplot regression function was used for model fitting.

calculated to be 2.3% at 48 hr).

The intracellular concentration reached maximum at 24 hr (Fig. 3), however, the antitumor effect of CKD-602 increased further on exposure for up to 72 hr (Fig. 1 and Fig. 2). These results indicate that the cumulative and delayed antitumor effects of CKD-602 are not due to slow drug uptake. At a drug concentration of 1250nM, 4hr exposure showed a significant difference in delayed effect compared to 24 hr, 48 hr, 72 hr and 96 hr. These results indicate that a certain level (ca. 3.5 μg/ml) of intracellular CKD-602 is required to obtain a delayed drug effect.

Relative importance of exposure time

The relationship between drug concentration (C), exposure time (T), and the resulting effect (h) of a chemotherapeutic agent can be expressed as $C^n \times T=h$ (Levasseur *et al.*, 1998). The value n, is derived from curve-fitting of the c versus t plot, and indicates the relative importance of concentration and exposure time. When $n > 1$, the drug concentration is more important for the drug effect, i.e., increased drug concentration has a greater impact than time, and vice versa.

Fig. 4 shows curve-fitting of the $C^n \times T=h$ plot of the IC_{50} of CKD-602 in HT-29 cells. The n value obtained was 0.481, indicating that the exposure time contributes more to the overall antitumor activity of CKD-602, as is the case for 9-aminocamptothecin (Li *et al.*, 2001). This means that drug exposure conditions with same $C \times T$ product may exhibit significantly different antitumor effects, and that greater effects are expected for longer exposures. The relative importance of exposure time can be attributed to the slow intracellular drug uptake as well as the delayed exhibition of cytotoxicity, as mentioned above based on the data shown in Figs. 1 to 3.

In summary, CKD-602 produced a cytotoxicity that was evident immediately after treatment (an immediate effect) and another after treatment was terminated (a delayed effect). CKD-602 treatment for 4hr was sufficient to induce a delayed cytotoxicity, however, longer treatment increased its cytotoxicity, which in part was due to the rather slow intracellular accumulation of drug, which required 24 hr to achieve its maximum equilibrium concentration. The anticancer activity of CKD-602 may be increased by a prolonged drug exposure of up to 72hr. In addition, $C^n \times T=h$ analysis indicated that increased exposure time may contribute more to the overall antitumor activity of CKD-602 than the drug concentration per se. Additional studies to determine details of the intracellular uptake kinetics (e.g., concentration dependency and retention study) and the cellular/molecular mechanism of the delayed and cumulative cytotoxicity of CKD-602 are warranted (Gieschke and Steiner 2000). Data regarding the concentration- and time-dependent antitumor activity of CKD-602 and changes in the drug concentration in cells when combined with systemic pharmacokinetic data in patients are very useful for identifying optimal treatment schedules, and hence, aid in designing *in vivo* studies including clinical trials (Kuh *et al.*, 1999 and 2000; van den Bongard *et al.*, 2000; Vigano *et al.*, 2001).

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