The Journal of Applied Pharmacology, 14, 90-95(2006)

# Pharmacodynamics of CKD-602 (Belotecan) in 3D Cultures of Human Colorectal Carcinoma Cells

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(Received 16 June, 2006; Accepted 28 June, 2006)

**Abstract** – CKD-602 exerts its antitumor effect via inhibition of topoisomerase I in cancer cells. Multicellular spheroid (MCS) and Multicellular layers (MCLs) are known as *in vitro* 3-dimensional models which closely represent tumor conditions *in vivo*. In order to investigate the potential of CKD-602 against human colorectal tumors, we evaluated the anti-proliferative activity and penetration ability of CKD-602 in MCS and MCL cultures of DLD-1 human colorectal cancer cells, respectively. The maximum effects ($E_{max}$) induced by CKD-602 were significantly lower in MCS compared to monolayers (48% *vs* 92%). With prolonged drug exposure, the $IC_{50's}$ of CKD-602 decreased to 23.5±1.0nM in monolayers after 24 h exposure and 42.3±1.7 nM in MCS after 6 days, respectively. However, no further increase in effect was observed for exposure time longer than growth doubling time (Td) in both cultures. Activity of CKD-602 was significantly reduced after penetration through MCL and also with cell-free insert membrane. In conclusion, CKD-602 showed significantly decreased anti-proliferative activity in 3D cultures (MCS) of human colorectal cancer cells. Tumor penetration of CKD-602 could not be determined due to loss of activity after penetration through cell free insert membrane, which warrants further evaluation using a modified model.

**Keywords** □ CKD-602, Belotecan, Pharmacodynamics, Multicellular spheroid, Multicellular layer, solid tumor, penetration

## INTRODUCTION

Camptothecin analogues are effective cytotoxic agents that mainly target topoisomerase I, an important nuclear enzyme for a variety of DNA functions including transcription and replication (Kohn and Prommier, 2000). Topotecan and irinotecan (CPT-11) have been approved by the FDA for the treatment of metastatic colorectal cancer, and advanced refractory ovarian cancer, respectively (Takimoto *et al.*, 1998). CKD-602 (7-[2-(N-isopropylamin) ethyl]-(20S)-camptothecin, Belotecan) is a new water soluble camptothecin analogue developed by Chong Kun Dang Pharmaceuticals, Seoul, Korea (Jew *et al.*, 1998). CKD-602 is four times more potent than topotecan with respect to inhibition of *in vitro* topoisomerase I enzyme activity (Lee *et al.*, 1998). CKD-602 has also shown activity against: ovarian, lung, colorectal and breast cancers (Lee *et al.*, 1998, 2000). KFDA (Korea food & Drug Administration) approved CKD-602 for the treatment of small cell lung cancer and ovarian cancer in 2003. There is a possibility to develop CKD-602 for the treatment of colorectal cancer.

Colorectal cancer is among the highly metastatic and resistant solid tumors to chemotherapy. Among the most active agents currently being used, irinotecan has shown a 50% response rate in patients refractory to 5-FU (Douillard *et al.*, 2000). However, development of new drugs and strategies are needed to improve clinical outcomes.

Data on the cytotoxic effects of new agents have been published based on the monolayer NCI-60 panel. However, the main cytotoxic agents, in current clinical use, were discovered using a mouse tumor model rather than an *in vitro* screening test (Baguley and Marshall, 2004). The 3D multicellular spheroid (MCS) culture model was first described by Sutherland *et al.*, 1971. The MCS model has many similarities to the *in vivo* solid tumor that include: abundance of extracellular matrix

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components, cell-cell interaction and cell-contact induced signal modification. Moreover, the structural resemblance of MCS (e.g., hypoxia and necrotic center) to tumor xenograft models has also been reported (Bordeau *et al.*, 1995; Dimanche-Boitrel *et al.*, 1998; Desoize and Jardillier, 2000). Because of these similarities, the 3D MCS has been extensively used in the field of cancer research. The penetration problem, of cytotoxic agents into tumor tissue, contributes to the poor clinical outcomes (Tannock *et al.*, 2002). Another 3D culture system, the Multicellular layers (MCLs), has been developed, and successfully used to study the penetration characteristics of a number of agents including paclitaxel and tirapazamine (Hicks *et al.*, 1998; Phillips *et al.*, 1999).

In order to provide more clinically relevant data for the development of CKD-602 against human colorectal cancers, we studied the pharmacodynamics of a promising agent CKD-602 (Belotecan) in the highly reliable *in vitro* 3D culture model of DLD-1 human colorectal adenocarcinoma cells.

## MATERIALS AND METHODS

### Materials

CKD-602 (Belotecan) was supplied from Chong-Kun Dang Pharmaceuticals (Seoul, South Korea). Agarose was purchased from Cambrex Bio-Science (Seaplaque®, Rockland, ME). The Transwell insert® (0.4 μm microporous membrane) was purchased from Corning Costar (Acton, MA). Methanethiosulfonate (MTS) was purchased from Promega (San Luis Obispo, CA). Sulforhodamine B and other reagents, unless otherwise stated, were purchased from Sigma (St. Louis, MO).

### Cell line and cell culture condition

The human colorectal adenocarcinoma cell line, DLD-1, was obtained from the Korean Cell Line Bank (Seoul, South Korea). DLD-1 cells were maintained in RPMI-1640 supplemented with 10% heat-inactivated FBS, penicillin (100 U/ml) and streptomycin (100 mg/ml) in a humidified 5% $CO_2$ cell culture incubator at 37°C.

### Culture of multicellular spheroid (MCS)

DLD-1 cells were grown as MCS using the liquid overlay technique as previously described (Frankel *et al.*, 2000) with some modification. Briefly, DLD-1 cells ($1\times10^4$ cells/100 μl) were seeded on agarose coated 96-well plates. On day 2, the plates were agitated at an interval of 15 min at 350 rpm using an orbital shaker. Media were replenished every other day. MCS growth was evaluated by size or MTS assay. For histological examinations, each MCS was fixed and embedded in paraffin, 5 μm sections were stained with H & E. For a semi-thin section, MCS was fixed and embedded in Epon 812, 2 μm sections were stained with toluidine blue.

### Cytotoxicity Assay

The sulforodamine B (SRB) assay was used for cytotoxicity assay in monolayers. DLD-1 cells in the log phase were harvested and plated in 96-well plates at a density of $2\times10^3$ cells/well. After a 24 h incubation, cells were exposed to drug-containing media for 72 h. Cells were then fixed with 10% trichloroacetic acid (ChemExper Chem, Lancaster, England) and then with 0.4% sulforhodamine B for 15 min. After five washes with 1 % acetic acid, protein-bound dye was extracted with 10 mM unbuffered Tris (Amresco, Solon, OH) and absorbance was measured at 540 nm. For the MCS system, cell viability was essentially assessed by MTS assay. Each spheroid was transferred to non-coated 96-well plate and subjected to CKD-602 treatment. At the end of the incubation period, 20 μl of MTS/PMS solution was added to each well and incubated at 37 °C according to the manufacturer's manual. After 4 h of incubation, absorbance was measured at 490 nm. The $IC_{50}$ was determined as the drug concentration required to reduce absorbance to 50% as compared to the control in each experiment.

### Culture of multicellular layers (MCLs)

DLD-1 cells were grown on collagen-coated microporous (0.4 μm) membranes in Transwell inserts at a plating density of $3\times10^5$ cells/100 μl. MCLs were grown for up to 5 days in a culture jar with continuous stirring using magnetic stirrer (Cowan *et al.*, 1996). For histological examinations, MCL inserts were fixed and embedded in paraffin, and 5 μm sections were stained with H & E. For a semi-thin section, MCLs were fixed and embedded in Epon 812, 2 μm sections were stained with toluidine blue.

### Drug penetration and bioassay

For the penetration study, MCLs were transferred from the culture jar to 6-well plates with the aid of a size adaptor. Drug containing media (100 μl) was loaded in the top chambers of each MCL, or cell free insert, and 7 ml fresh media was supplemented in the bottom chamber. After 24 h penetration through MCL, or the cell free insert, media in the bottom chamber was transferred to 96-well plates in which DLD-1 cells were seeded at a density of $2\times10^3$ cells/well. Cells were also exposed to

fresh media containing CKD-602 at concentrations calculated assuming 100% penetration. SRB assay was used to measure viability of cells exposed to fresh and conditioned drug containing media for 72 h.

### Data analysis

The % growth inhibition, maximum growth inhibition ($E_{max}$) and $IC_{50}$ were calculated as using Eq. 1 and Eq.2.

$$\% \text{ Growth inhibition} = \left[1 - \left(\frac{\text{mean absorbance of treated cells}}{\text{mean absorbance of control cells}}\right)\right] \times 100 \quad \text{(Eq.1)}$$

$$\% \text{ Growth inhibition} = (100 - R) \times \left(1 - \frac{[D]^m}{K_d^m + [D]^m}\right) + R \quad \text{(Eq.2)}$$

Where D is the drug concentration, $K_d$ is the drug concentration that produces a 50% reduction in cell growth (i.e., $IC_{50}$), $m$ is the Hill-type coefficient, and R is the residual unaffected fraction (the resistance fraction).

## RESULTS

### Growth of DLD-1 MCS

DLD-1 cells formed a fully compacted and well-rounded spheroid and showed a steady growth pattern over a period of 10 days, reaching a diameter up to 800 μm. The overall doubling time ($T_d$) was calculated to be about 6.2 days for MCS compared to 0.9 day for monolayers. In the semi-thin section of DLD-1 MCS grown for 3 days (diameter of 490 μm), tightly packed cells with no necrotic or apoptotic region were observed (Fig. 1). In addition, MCS of DLD-1 showed biphasic growth patterns, i.e., initial growth rate during the first 4 days was slower with marked contrast to a later fast growth days (Fig. 2).

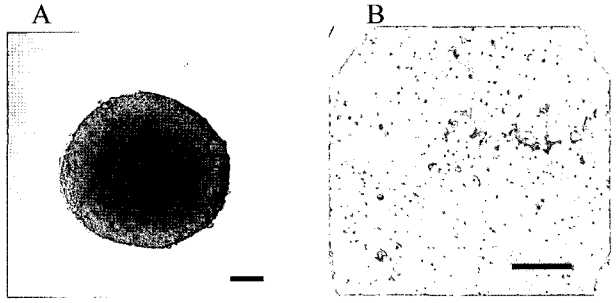


**Fig. 1.** Morphology of multicellular spheroids of DLD-1 human colon cancer cells grown for 3 days. Phase contrast micrograph (A) and semi-thin sections (B) of DLD-1 MCS. Scale bars indicate 100 μm.

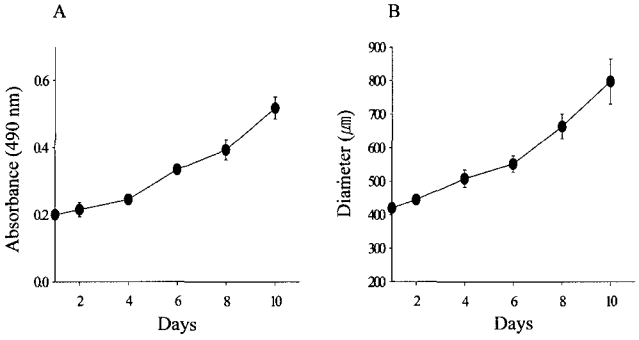


**Fig. 2.** The growth of MCS of DLD-1 cells determined using MTS assay (A) and diameter (B) up to 10 days after seeding on agarose-coated 96 well plates.

### Antiproliferative effects of CKD-602

As shown in Table I and Fig. 3, the MCS of DLD-1 showed significant resistance to CKD-602 compared to the monolayers. The maximum growth inhibition ($E_{max}$) was found to be 92% for the monolayers, but only 48% for the MCS. The $IC_{50}$

**Table I.** A pharmacodynamic parameter of cytotoxicity of CKD-602 in DLD-1 cells grown in monolayers or multicellular spheroids (MCS)

| | | | | | | |
|---|---|---|---|---|---|---|
| Monolayers | Parameter | Exposure duration (h) | | | | |
| | | 4 | 10 | 24 | 48 | 72 |
| | $IC_{50}$ (nM) | 97.4† | 72.2±7.7 | 23.5±1.0 | 20.5±1.2 | 16.5±0.6 |
| MCS | Parameter | Exposure duration (days) | | | | |
| | | 1 | 2 | 3 | 6 | 9 |
| | $IC_{50}$ (nM) | 296±84.5 | 187±15.9 | 92.9±4.1 | 42.3±1.7 | 41.4±4.9 |

Each Value represents the mean±SD of three independent experiments.
†obtained in a single experiment.

of CKD-602 in the monolayers exposed for 72 h was 16.5± 0.6 nM, and 41.4± 4.9 nM in MCS exposed for 9 days. Although the $IC_{50's}$ decreased with longer exposure times, drug exposure longer than $T_d$ did not induce further decrease in both systems (Table I, Fig. 3).

### Growth of DLD-1 MCLs

DLD-1 grown as MCL on collagen coated membrane showed a steady growth up to 5 days. At the 5th day of growth, the MCL of DLD-1 showed 150 μm of thickness as measured by the semi-thin sections. On the other hand, the MCL thickness was 65 μm when observed with paraffin embedded sections. The shrinkage of thickness, in a paraffin embedded sample, coincided with the morphological differences between the two histological processing techniques. Cells were round and heterogeneously stained in semi-thin sections of MCL, however, spindle shaped cells were seen with paraffin embedded sections (Fig. 4).

### CKD-602 penetration through DLD-1 MCL

Activity of CKD-602, after 24 h penetration through the DLD-1 MCL or the cell free insert membrane was evaluated. The drug concentrations loaded in the top chambers were 135, 230, and 335 nM, based on the maximum plasma levels observed in clinical studies, and another two higher concentrations, 500 and 700 nM. The highest concentration (700 nM) showed viability around 20% when diluted by 70 folds assuming 100% penetration. Decrease in anti-proliferative activity, up to 35%, was observed after penetration through the cancer cell layers, i.e., survival rates increased from about 25 to 60% at 700 nM. It was noted that, however, a similar decrease in anti-proliferative activity was observed after penetration through cell free insert (Fig. 5).

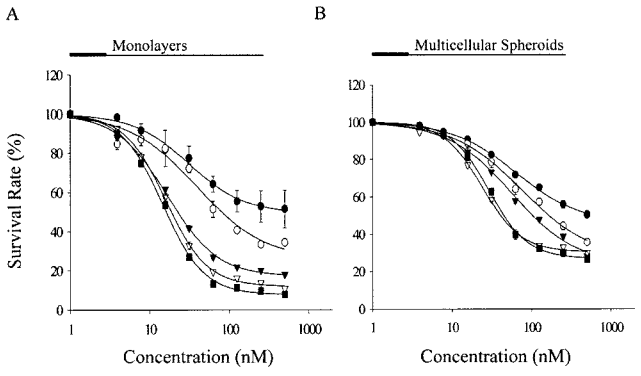


**Fig. 3.** Representative dose-response curves of CKD-602 following various drug exposure times in monolayers (A) and MCS (B) of DLD-1 cells. Symbols are ●, 4 h; ○, 10 h; ▼, 24 h; ▽, 48 h; ■, 72 h, respectively, in monolayers, and ●, 1day; ○, 2 day; ▼, 3 day; ▽, 6 day; ■, 9 day, respectively, in

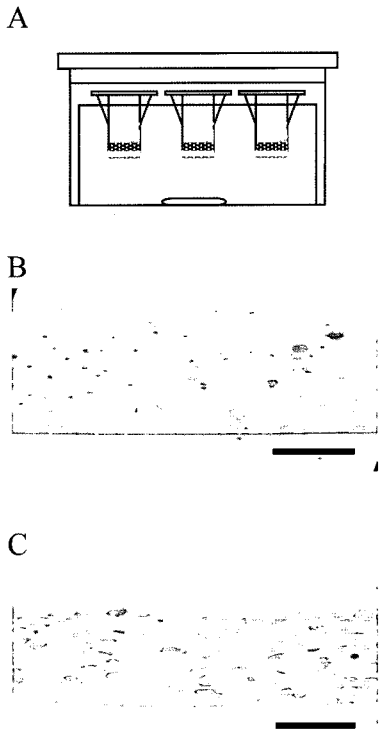


**Fig. 4.** A schematic picture of the MCL culture jar (A) and representative histological sections of DLD-1 multicellular layers (MCLs) grown for 5 days. Sections were obtained using epon-block (semi-thin section) (B) and paraffin block (C), respectively.

## DISCUSSION

CKD-602 is a promising new topoisomerase I interactive agent and showed activity against human colorectal cancers in screening studies *in vitro*. It has demonstrated encouraging activity in several cancer cell lines including colorectal cancers (Park *et al.*, 2002). The 3D *in vitro* culture system closely resembles *in vivo* tumor conditions; where cellular resistance and a penetration barrier to cytotoxic agents represent the major obstacles to obtain full efficacy (Tannock *et al.*, 2002). Therefore, in the current work, we evaluated the anti-proliferative effects of CKD-602 in MCS of human colorectal cancer cells. Furthermore, we examined the post-penetration activity of CKD-602 using MCLs.

The cellular environments of *in vivo* solid tumors play a major role in physiology and sensitivity to anticancer agents.

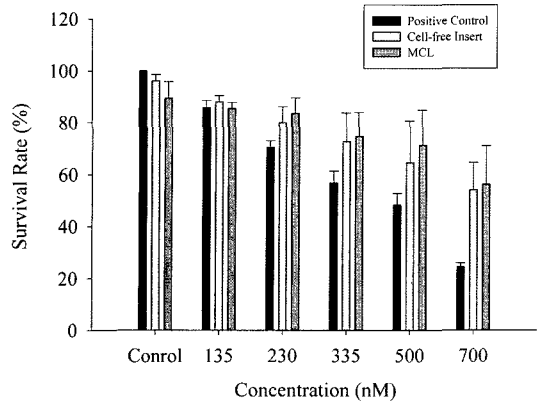


**Fig. 5.** Anti-proliferative activities of CKD-602 after penetration through the Transwell insert® membrane with or without multicellular layers. Media containing various concentrations (135, 230, 335, 500, and 700 nM) was loaded in top chambers. After 24 h penetration through MCLs, or the cell free insert, media in the bottom chamber were carefully procured, and then re-treated in 96-well plates. Freshly prepared drug solution prepared assuming 100% penetration (70 times dilution) was used as a positive control.

Cell-cell and cell-extracellular matrix (ECM) contacts are necessary for many biological functions such as angiogenesis, differentiation, apoptosis as well as cell proliferation (Galmarini and Galmarini, 2003; Hazlehurst *et al.*, 2003). Monolayer cultures, the most common experimental model for studying drug effects, have a major shortcoming in that cells do not mimic the complex and heterogeneous environmental properties of solid tumors *in vivo*. As a result, the data obtained from these experimental models lead to incorrect predictions of drug efficacy when drugs are tested in clinical trials. MCS may be considered as a suitable preclinical model because some of the complex features of solid tumors could be recapitulated by interactions of the tumor cells.

DLD-1 grown as MCS showed an exponential growth rate over the examined period. It is well known that the ability of cancer cells to form a 3D structure mainly depends on producing its own ECM components. In addition, cell tight junctions have been shown to enhance aggregation ability (Bates *et al.*, 2000). In fact, we have observed that DLD-1 cells produce their own fibronectin when forming a multicellular structure (data not shown). Our previous data, obtained by TEM images, also clearly showed the presence of desmosomes in MCS of DLD-1 (Lee *et al.*, 2004). These structural similarities of MCS to *in vivo* solid tumor microenvironments support clinical relevance of cytotoxicity data of the 3D MCS model.

The results of our study showed that the MCS was more resistant to CKD-602 than the monolayers. $IC_{50's}$ were drastically lower under monolayer conditions compared to MCS at all drug exposure times. However, in both culture conditions, the $IC_{50}$ value did not significantly change when treated for longer times than $T_d$. Moreover, our present data clearly demonstrated that the maximum effect of CKD-602 in MCS was poor ($E_{max}$ 48% after 9 days exposure) compared to that in monolayers ($E_{max}$ 92 % after 3 days exposure). $IC_{50's}$ decreased with longer exposure times up to $T_d$ (Table I, Fig. 3), hence, antiproliferative activity of CKD-602 can be enhanced with increasing exposure time up to the tumor doubling time. Therefore, these data may be useful for protocol development in clinical trials.

Multilayer structure-related chemoresistance is well documented. A few previous studies have shown that PTX was significantly less cytotoxic when tumor cells, as a small multicellular aggregate, were exposed to PTX. Spheroids of MCF-7 human breast carcinoma cells were significantly less sensitive to PTX than the monolayers (Nicholson *et al.*, 1997). Moreover, it is well known that chemotherapeutic resistance associated three-dimensional tumor structure results from a decreased growth rate, decreased drug penetration as well as induction of hypoxia. Cell-to-cell interactions in the three-dimensional tumor microenvironments could lead to a phenotypic switch related with changes in tissue organization, altered expression of integrins, and increase expression of ECM components (Galmarini and Galmarini, 2003). These may result in alteration of signal transduction related to apoptosis and/or drug resistance.

In order to better understand the different sensitivity of CKD-602 in two different culture systems, we evaluated the penetration ability of CKD-602 using MCLs, another validated 3D model. Discordance of MCL thickness was observed between the semi-thin and paraffin embedded sections which has been previously reported (Sutherland *et al.*, 1986). However, these results have no effect on the model validity for the CKD-602 penetration study. The post-penetration activities of CKD-602 were considerably decreased compared to freshly made positive controls (Fig. 5). These results suggest that CKD-602 has a penetration problem in gaining entry into the multilayer of tumor cells. However, it was noted that there was no significant difference in the post-penetration activity of CKD-602 between the cell free insert and the MCLs. No reduction of CKD-602 activity was observed after incubation in culture medium for 24 h at 37°C(data not shown), suggesting that irreversible protein binding was not the cause for the loss of

CKD-602 activity. Therefore, further investigation and modification of the penetration study using MCL model are needed with consideration of adsorption to membranes and other possible causes.

In conclusion, CKD-602 showed significantly decreased anti-proliferative activity in 3D cultures (MCS) of human colorectal cancer cells. Tumor penetration of CKD-602 could not be determined due to loss of activity after penetration through cell free insert membrane, which warrants further evaluation using a modified model.

## ACKNOWLEDGEMENTS

This work was supported by a grant from the Ministry of Science & Technology (M10414030001-05N1403-001-00111), Republic of Korea.

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