Investigation into the Efficacy of Val-SN-38, a Valine-Ester Prodrug of the Anti-Cancer Agent SN-38

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Abstract

We recently reported that Val-SN-38, a novel valine ester prodrug of SN-38, had greatly improved the intracellular accumulation of SN-38 in MCF-7 cell line, probably through enhanced uptake via amino acid transporters. In the present study, the efficacy of Val-SN-38 was further investigated both \textit{in vitro} and \textit{in vivo}. It was found that the \textit{in vitro} cytotoxic effect of Val-SN-38 was similar to that of SN-38. Moreover, Val-SN-38 exhibited an equal potency to that of SN-38 in survival experiments \textit{in vivo}. Because these results seemed to be contrary to the previous finding, further investigation was performed to find out the underlying cause of the contradiction. As only the lactone form is known to have cytotoxic activity, the proportion of lactone in Val-SN-38 and SN-38 was determined, but no differences were found. However, it turned out that Val-SN-38 had poor stability compared with SN-38, which resulted in a decrease in beneficial efficacy for Val-SN-38. Overall, the present study showed that a valine-added prodrug approach could be advantageous provided that the stability of the compound can be ensured. We believe this is a noteworthy study that unravels the discrepancy between intracellular accumulation and efficacy of valine-added prodrug.

Key Words: Val-SN-38, SN-38, Irinotecan, Ester prodrug, Efficacy, Stability

INTRODUCTION

SN-38 is a cytotoxic compound metabolized from irinotecan, which is a topoisomerase inhibitor that is used for various cancer treatments. In particular, SN-38 garnered much interest when it was found to be 100-fold more potent than irinotecan in terms of \textit{in vitro} cytotoxicity (Kawato \textit{et al.}, 1991). The pharmacological activity of these camptothecin derivatives derives from the α-hydroxy-δ-lactone ring structure, which is essential for the stabilization of a DNA-topoisomerase complex (Mullangi and Srinivas, 2009).

However, many anti-cancer agents suffer from a reduction in efficacy, because they are often pumped out of the cell by various efflux transporters. This is particularly true with camptothecin derivatives, since both SN-38 and irinotecan are good substrates of P-glycoprotein (P-gp), which is one of the most popular efflux transporters (Iyer \textit{et al.}, 2002; Itoh \textit{et al.}, 2005). Therefore, many researchers have attempted to bypass the effect of these efflux transporters, but it has proven to be very difficult to directly control the nature of the efflux transporters. Thus, a “workaround” approach can be applied to increase cellular uptake. In this context, a prodrug approach that adds a valine moiety to a parent compound such as valacyclovir (Balimane \textit{et al.}, 1998; Ganapathy \textit{et al.}, 1998; Guo \textit{et al.}, 1999) and valganciclovir (Sugawara \textit{et al.}, 2000; Umapathy \textit{et al.}, 2004) has gained much interest, because it successfully increased the oral bioavailability of the parent drugs via various amino acid transporters in the uptake process. Based on this idea, we have synthesized a prodrug of SN-38 called Val-SN-38, which is a valine-ester of SN-38, and found that, when compared with SN-38, Val-SN-38 exhibited a 5.4-fold increase in intracellular concentration, which was mostly attributed to amino acid transporters (Kwak \textit{et al.}, 2012).

However, one possible problem with the efficacy of Val-SN-38 is that valine is added by an ester bond, which could be vulnerable to various endogenous esterases (Khan \textit{et al.}, 2000; Mathijssen \textit{et al.}, 2001). Furthermore, SN-38 has a unique characteristic — a process known as \textit{interconversion} between two forms: carboxylate and lactone. This causes efficacy problems because only the lactone form possesses cytotoxic activity, while the carboxylate form is regarded as inactive (Hertzberg \textit{et al.}, 1990). Moreover, this interconver-
sion is governed by many microenvironmental factors such as pH (Fassberg and Stella, 1992), ionic strength (Fassberg and Stella, 1992), and even protein concentration (Burke and Mi, 1993). Thus, an investigation into the final efficacy of Val-SN-38 is necessary in order to verify the overall advantage of valine addition to SN-38.

Therefore, in the present study, the efficacy of Val-SN-38 was investigated under both in vitro and in vivo conditions, and estimations were made as to whether there were any stability issues regarding Val-SN-38.

MATERIALS AND METHODS

Reagents

Irinotecan was purchased from Sigma-Aldrich (St. Louis, MO, USA). Val-SN-38 and SN-38 were obtained by previously mentioned methods (Kwak et al., 2012). Stocks of anti-cancer agents (10 mM) were prepared in dimethyl sulfoxide (DMSO) and stored in -80°C. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was purchased from Armco Inc. (Solon, OH, USA), and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Cell lines and cytotoxicity test

MCF7, CT26 and HT29 cell lines were obtained from the American Type Culture Collections (Manassas, VA, USA). Cell culture media and fetal bovine serum (FBS) were purchased from Hyclone (Logan, UT, USA) and Sigma-Aldrich, respectively. Cells were cultured under 10% FBS and DMEM containing 100 unit/ml penicillin and 0.1 mg/ml streptomycin. All cell lines were split around 80% confluence, and cultured under 95% humidity and 5% CO2 at 37°C.

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Cells were seeded with 1×10^5 cells/well on 96-well plates and cultured for 24 h. After cells had reached 80% confluence, drugs were treated on each well for 72 h. Cytotoxicity was then measured using a standard MTT assay method. Briefly, 0.5 mg/ml of MTT dissolved in serum-free media was treated on each well for 2 h. Later, absorbance was read using a microplate reader (Triad LT, Dynex Technologies Inc, Chantilly, VA, USA) at 540 nm wavelengths.

Colon cancer animal model and in vivo survival test

A colon cancer model was introduced via a surgical orthotopic implantation method (Hoffman, 1999). Briefly, mice were anesthetized and the cecum was exteriorized by laparotomy. μl of CT26 cell suspension (1×10^6 cells) was injected into the cecal wall. After injection, the gut was returned into the abdominal cavity and the incision was closed. Designated drugs were administered intravenously and the in vivo survival rate was observed during the experiment period. All animal study protocols were approved by the Animal Care and Use Committee of the College of Pharmacy, Seoul National University.

Stability test

The stability of SN-38 and Val-SN-38 was investigated in PBS, MCF7 cell lysates, and rat plasma. Specifically, MCF7 cell lysates were obtained via the following method. After MCF7 cells were harvested, phenylmethylsulfonyl fluoride (PMSF, Sigma-Aldrich, St. Louis, MO, USA) was added and ultrasonication was performed for 10 sec. Lysates were then obtained by supernatants, which were acquired by centrifugation at 4°C and 13,200 rpm for 15 min. 5 μM of SN-38 and Val-SN-38 was dissolved in designated solutions and vortexed for 3 min. 50 μl of samples were acquired at various time points in a 37°C water bath, and were added into 150 μl of ice-cold acetonitrile and vortexed again for 5 min. Mixtures were then centrifuged for 5 min at 13,200 rpm, and 100 μl of supernatants were mixed with 200 μl of 10 mM K2HPO4 (pH 7.4) and then 100 μl of the mixture was analyzed by HPLC using a C18 analytical column (250×4.6 mm, C18, Varian, Inc., Santa Clara, CA, USA). The mobile phase was composed of 10 mM KH2PO4 (pH 7.4) and acetonitrile at a ratio of 62:38, respectively. The flow was maintained at 1 ml/min. For the fluorescence detection of the amount of SN-38, wavelengths of 380 and 540 nm were used for excitations and emissions, respectively.

Statistics

All data were shown as the mean ± standard deviation. Graphs and statistical analyses were produced by Graphpad Prism (San Diego, California, USA, www.graphpad.com).

RESULTS

In vitro cytotoxicity of Val-SN-38

To identify the effect of Val-SN-38, an in vitro cytotoxicity test was performed in MCF7 cells, and the potency was compared with that of SN-38 through MTT assay. As shown in Fig. 1, the 2 compounds exhibited similar sigmoidal dose-response curves, with no apparent difference in terms of the EC50 (SN-38: 0.248 μM [0.147-0.417 μM, 95% Confidence intervals (CI)] vs. Val-SN-38: 0.450 μM [0.250-0.811 μM, 95% CI], n=3). Considering our previous report that accumulation of Val-SN-38 in MCF7 was much higher than that of SN-38 (Kwak et al., 2012), this result appeared to contradict our expectations. To verify if this result was a phenomenon limited to MCF7 cells, we further performed similar MTT assays in different cancer cell lines, namely CT26 and HT29, with irinotecan as a positive control. As a result, however, MTT assay in CT26 revealed that the EC50 of Val-SN-38 (52.1 μM [33.4-81.4 μM, 95% CI], n=3) was slightly higher when compared with that of SN-38 (17.3 μM [11.1-26.9 μM, 95% CI], n=3), suggesting that the potency of Val-SN-38 was weaker (Fig. 2A). Likewise, MTT assay in HT29 also showed less potency for Val-SN-38 (9.18 μM [3.82-22.1 μM, 95% CI], n=3) compared with that