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

Eun-Young Kwak¹, Min-Koo Choi², Su-Geun Yang³,⁴, Chang-Koo Shim¹ and Won-Sik Shim⁵*¹

College of Pharmacy, ¹Seoul National University, Seoul 151-742; ²Dankook University, Cheonan 330-714, ³Clinical Research Center, School of Medicine, Inha University, Incheon 400-712, ⁴Utah-Inha DDS and Advanced Therapeutics, Incheon 406-840, ⁵College of Pharmacy, Gachon University, Incheon 406-799, Republic of Korea

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 in vitro and in vivo. It was found that the 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 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 in vitro cytotoxicity (Kawato et al., 1991). The pharmacological activity of these camptothecin derivatives derives from the α-hydroxy-ß-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 et al., 2002; Itoh 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 et al., 1998; Ganapathy et al., 1998; Guo et al., 1999) and valganciclovir (Sugawara et al., 2000; Umaphathy 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 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 (Khanna et al., 2000; Mathijssen et al., 2001). Furthermore, SN-38 has a unique characteristic — a process known as 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 et al., 1990). Moreover, this interconver-
tion 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 K₂HPO₄ (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

![Fig. 1. Cell viability tests in MCF7 cell line. Notice the similar cytotoxicity of Val-SN-38 (empty square) and SN-38 (filled circle). Error bars indicate SD.](image-url)
of SN-38 (1.32 μM [0.66-2.63 μM, 95% CI], n=3) (Fig. 2B). Therefore, these data indicate that although both compounds were much more potent than the parent compound irinotecan (Fig. 2), Val-SN-38 seemed to exhibit no apparent advantages over SN-38 in terms of \textit{in vitro} cytotoxicity in various cell lines. However, MTT assay may simply represent the intrinsic potency of compounds rather than overall efficacy in \textit{vivo}, because it does not take other factors, such as pharmacokinetics (e.g., initial uptake rate), into consideration. Therefore, \textit{in vivo} survival tests were conducted on cancer-induced mice because conclusions for the merit of Val-SN-38 should not be made solely by MTT assays.

\textbf{In vivo survival test of Val-SN-38 in colon cancer-induced mice}

To further test if Val-SN-38 has an \textit{in vivo} anti-cancer effect, colon cancer-induced mice were used and their survival rates were investigated. As shown in Fig. 3, the survival rate of the irinotecan-administered group was the worst of the 3 compounds, since all mice in the group failed to live 28 days (n=4). Otherwise, mice administered Val-SN-38 (n=4) and SN-38 (n=4) survived longer than the irinotecan-administered group by at least 2 weeks, suggesting both Val-SN-38 and SN-38 exhibited a stronger \textit{in vivo} anti-tumor effect than irinotecan. Otherwise, the survival rate between Val-SN-38 and SN-38 was indistinguishable. This appears to be in line with the aforementioned \textit{in vitro} cytotoxicity results (Fig. 1, 2), suggesting that Val-SN-38 had no advantage over SN-38 in terms of \textit{in vivo} efficacy.

\textbf{Measurement of lactone proportions in Val-SN-38 and SN-38}

Because the unsatisfactory results of Val-SN-38 were observed under both \textit{in vitro} and \textit{in vivo} conditions, we further searched for the cause of the issue. First we investigated whether the problem stemmed from the conformational interchange, a process known as interconversion. As shown in Fig. 4, both Val-SN-38 and SN-38 were expected to have 2 interchangeable structures - namely, carboxylate and lactone forms. The problem was that only the lactone form holds the cytotoxic effect due to its α-hydroxy-lactone ring structure. Therefore, the cytotoxic effect was expected to be proportional to the total amount of the lactone form. Besides, because Val-SN-38 is a prodrug form of valine-added SN-38, it should have been easily hydrolyzed into SN-38 by esterase, which could have complicated the final equilibrium among these conformations. We measured the proportions of the total lactone forms of both SN-38 and Val-SN-38 under various conditions.

As shown in Fig. 5A, the proportions of the lactone form in SN-38 continued to decrease during 120 min in all 3 condi-
Noticeably, the initial proportion of the lactone form was very high (80-90%) in all 3 conditions, suggesting a predominance of the lactone form. There was then a fast reduction phase within the first 15 min, which was followed by a slow reduction phase. Remarkably, the proportion of the lactone form of SN-38 in plasma at 120 min was the lowest (~30%), while those in PBS and MCF7 lysates were around 40%. In the case of Val-SN-38, slightly lower initial proportions of the lactone form (60-70%) at early time points were found in all 3 conditions (Fig. 5B). A similar overall declining pattern was seen in Val-SN-38, falling in a range of from 30-40% for the lactone form at 120 min (Fig. 5B). Therefore, with the exception of a slight difference in proportions at the early time points, the proportions of lactone in both SN-38 and Val-SN-38 were similar.

Stability test of Val-SN-38 and SN-38

We further tested for stability problems with Val-SN-38 and SN-38. As shown in Fig. 6A, SN-38 in PBS seemed to be relatively stable; nearly 80% remained after 120 min. Even in plasma, SN-38 was maintained at about 60% and reached a plateau. Although the stability in MCF7 lysates seemed rather unstable, this was expected considering various enzymes and/or cell contents were present in the lysate.

However, Val-SN-38 broke down quite rapidly when dissolved in PBS, and only 50% remained after 120 min (Fig. 6B). This situation worsened when the drug was dissolved in MCF7 lysates and plasma, since merely 40% of the compound was left after 120 min, and no plateau was reached even after 120 min (Fig. 6B). These results clearly showed a severe stability issue for Val-SN-38 by comparison with SN-38.

As a summary, the present study showed that the efficacy of Val-SN-38 was not an improvement over SN-38, which was corroborated by both in vitro (Fig. 1, 2) and in vivo experiments (Fig. 3). Because only the lactone form is active (Fig. 4), the lactone proportions in Val-SN-38 and SN-38 were measured, and no large conformational differences were found between the 2 compounds (Fig. 5), but Val-SN-38 was found to be more unstable than SN-38 (Fig. 6). Considering that the intracellular accumulation of Val-SN-38 was greatly increased compared to SN-38 (Kwak et al., 2012), the efficacy of Val-SN-38 would have been higher than SN-38 if there were no stability issues. Therefore, it was concluded that the instability of Val-SN-38 resulted in reduced (similar) efficacy, even though the intracellular accumulation of Val-SN-38 was much higher than SN-38. Therefore, the present study strengthens the idea that the valine-added prodrug approach can be truly successful in terms of efficacy, provided that the stability of the compound is guaranteed.

DISCUSSION

Irinotecan (CPT-11) is a potent topoisomerase I inhibitor, which metabolizes into the active metabolite SN-38, exert-
ing broad spectrum of anti-cancer activity such as colorectal, lung, esophageal, gastric, cervical and ovarian cancers (Rothenberg, 2001). One of the major side effects of SN-38 is the severe diarrhea, as a result of direct enteric injury (Araki et al., 1993). Other side effects such as neutropenia, vomiting, alopecia, asthenia, and nausea have been reported (Cunningham et al., 1998).

In our previous study, we reported that Val-SN-38 - a valine ester of SN-38 showed a greatly increased intracellular accumulation compared to SN-38 (about 5-fold), suggesting the possible benefits of its use in terms of efficacy (Kwak et al., 2012). The present study was thus performed to determine whether Val-SN-38 has any advantages over SN-38 in terms of usage. Unfortunately, Val-SN-38 showed no improvement in efficacy over SN-38 under either in vitro or in vivo conditions. As shown in Figs. 1 and 2, the in vitro cytotoxic effect of Val-SN-38 was similar when compared with that of SN-38. Moreover, an in vivo survival test also failed to demonstrate any benefits of Val-SN-38 over SN-38 (Fig. 3). However, considering the dramatically increased intracellular uptake of Val-SN-38 in the previous study, these results were not only unsatisfactory but also a bit paradoxical. Therefore, the study was extended to unravel the discrepancy between accumulation and efficacy of Val-SN-38.

As mentioned earlier, only the lactone form is known to hold stronger cytotoxic activity, as lactone is necessary for topoisomerase binding (Hertzberg et al., 1990). In fact, this process is largely governed by microenvironmental conditions such as pH (Fassberg and Stella, 1992), ionic strength (Fassberg and Stella, 1992), and even protein concentrations (Burke and Mi, 1993). In particular, pH might have played a role in the present study, because the lactone form is less prevalent at neutral conditions (Rivory et al., 1994), under which most of our experiments were performed (pH 7). Indeed, our results showed that there was a lower proportion of lactone forms both in Val-SN-38 and SN-38, where only 30-40% of lactone forms were present at 120 min under all 3 conditions (Fig. 5). In fact, this is consistent with the previous results showing that the percentage of SN-38 lactone remaining in sodium phosphate (pH 7) was around 40% at 120 min (Tallman et al., 2005). Therefore, it could be estimated that neutral pH conditions might have potentiated the overall lower proportion of the lactone form in all situations.

One interesting result was that the initial proportions of the lactone forms in Val-SN-38 were much lower than that of SN-38 under all 3 conditions. In other words, the carboxylate form predominated in the initial phase of Val-SN-38 when compared to SN-38. This phenomenon may extend to an assumption that uptake transporters might have favored the carboxylate over the lactone form as a substrate. For instance, OATP1B1 and OATP1B3 uptake transporters favor the carboxylate form of AR-67, which is an SN-38 derivative, whereas efflux transporters like P-gp and BCRP favor the lactone form (Adane et al., 2010). Therefore, we cannot exclude the possibility that the carboxylate form of Val-SN-38 was favored by relevant amino acid transporters like ATA1, ATA2, and ATB1+, resulting in a significant accumulation increase, although further verification is necessary.

Furthermore, we cannot rule out the possibility that efflux transporters also favor the carboxylate form, ultimately leading to low intracellular levels. Indeed, there already is a report that only the carboxylate form of SN-38 is transported via Mrp2 in the rat liver (Chu et al., 1997). Likewise, Scott et al. reported that a much higher plasma level of 20(S)-camptothecin lactone was observed compared with the plasma level of carboxylate, suggesting that the carboxylate form was more highly excreted into urine and bile in rats (Scott et al., 1994). In fact, Mrp2 favored Val-SN-38 more than SN-38 in our previous study, although P-gp, BCRP, and MRP1 did not (Kwak et al., 2012). Given that the previous accumulation experiments were performed only after a 30 min incubation (Kwak et al., 2012), there might have been insufficient time for efflux transporters like P-gp, BCRP and MRP1 to carry the compound out of the cell in the previous study. Again, these possibilities require a more thorough inspection.

However, although it is likely that the lactone/carboxylate interconversion may affect the transport process by various uptake and/or efflux transporters, it should be noted that the final lactone proportion of Val-SN-38 was very similar to that of SN-38 (Fig. 5). The fact that Val-SN-38 is more highly accumulated than SN-38, suggests that Val-SN-38 is more prone to degrade than SN-38, leading to equilibrium within in a similar intracellular concentration range, and, thus, a comparable efficacy. In other words, the poor stability of Val-SN-38 offsets its enhanced uptake, and results in no beneficial efficacy. Thus, this possibility was investigated further.

As a result, SN-38 was found to be reasonably stable in PBS, but the compound became slightly unstable in plasma (Fig. 6A). It seemed more unstable with MCF7 lysates, since it failed to converge into a plateau even at 120 min (Fig. 6A). On the other hand, Val-SN-38 in all 3 conditions showed a more deteriorated stability, and more than half of the compound was degraded in all 3 conditions at 120 min (Fig. 6B). Therefore, the stability of Val-SN-38 was indeed less than that of SN-38. Taken together, these results support the possibility that the instability of Val-SN-38 might be the reason for its low efficacy.

To review the entire process of Val-SN-38 treatment, the compound offers great advantage in the uptake process with the aid of some amino acid transporters (Kwak et al., 2012). However, because of its poor stability profile, it seems that the increased accumulation of Val-SN-38 is offset by its rapid degradation, resulting in an efficacy similar to SN-38.

Overall, the reason for the unsatisfactory efficacy of Val-SN-38 over SN-38 seems due largely to its unstable manner. We believe the present study is a noteworthy one that unravels the discrepancy between intracellular accumulation and efficacy of valine-added prodrug. This further implies that Val-SN-38 is not a good model compound to promote the idea of a “valine-added prodrug anti-cancer agent.” That is not because it failed to show better efficacy over SN-38, but because it involves too many factors and/or reactions to consider. It would be interesting to introduce a valine moiety to other anti-cancer compounds, which are relatively stable and do not undergo a chemical process such as conformational interconversion. Moreover, it would be interesting to introduce valine to SN-38 with more stable linkage such as amide, which may improve both accumulation and efficacy. Therefore, the present study suggests that a thorough and in-depth consideration is mandatory in order to improve the efficacy of anti-cancer agents using the prodrug approach. These results also support the theory that a valine-added prodrug approach will be effective for stable compounds.
REFERENCES

Adane, E. D., Liu, Z., Xiang, T. X., Anderson, B. D. and Leggas, M. (2010) Factors affecting the in vivo lactone stability and systemic clearance of the lipophilic camptothecin analogue AR-67. *Pharm. Res.* **27**, 1416-1425.

Araki, E., Ishikawa, M., ligo, M., Koide, T., Itabashi, M. and Hoshi, A. (1993) Relationship between development of diarrhea and the concentration of SN-38, an active metabolite of CPT-11, in the intestine and the blood plasma of athymic mice following intraperitoneal administration of CPT-11. *Jpn. J. Cancer Res.* **84**, 697-702.

Balimane, P. V., Tamai, I., Guo, A., Nakashishi, T., Kitada, H., Leibach, F. H., Tsuji, A. and Sinko, P. J. (1998) Direct evidence for peptide transporter (PepT1)-mediated uptake of a nonpeptide prodrug, valacyclovir. *Biochem. Biophys. Res. Commun.* **250**, 246-251.

Burke, T. G. and Mi, Z. (1993) Preferential binding of the carboxylic form of camptothecin by human serum albumin. *Anal. Biochem.* **212**, 285-287.

Chu, X. Y., Kato, Y., Niinuma, K., Sudo, K. I., Hakusui, H. and Sugiya, M. (1997) Multispecific organic anion transporter is responsible for the biliary excretion of the camptothecin derivative irinotecan and its metabolites in rats. *J. Pharmacol. Exp. Ther.* **281**, 304-314.

Cunningham, D., Pyrhönen, S., James, R. D., Punt, C. J., Hickish, T. F., Heikkila, R., Johannesen, T. B., Starkhammar, H., Topham, C. A., Awad, L., Jacques, C. and Herail, P. (1998) Randomised trial of irinotecan plus supportive care versus supportive care alone after fluorouracil failure for patients with metastatic colorectal cancer. *Lancet* **352**, 1413-1418.

Fassberg, J. and Stella, V. J. (1992) A kinetic and mechanistic study of the hydrolysis of camptothecin and some analogues. *J. Pharm. Sci.* **81**, 676-684.

Ganapathy, M. E., Huang, W., Wang, H., Ganapathy, V. and Leibach, F. H. (1998) Valacyclovir: a substrate for the intestinal and renal peptide transporters PEPT1 and PEPT2. *Biochem. Biophys. Res. Commun.* **246**, 470-475.

Guo, A., Hu, P., Balimane, P. V., Leibach, F. H. and Sinko, P. J. (1999) Interactions of a nonpeptidic drug, valacyclovir, with the human intestinal peptide transporters PEPT1 and PEPT2. *Biochem. Biophys. Res. Commun.* **250**, 246-251.

Hertzberg, R. P., Busby, R. W., Caranfa, M. J., Holden, K. G., Johnson, R. K., Hecht, S. M. and Kingsbury, W. D. (1990) Irreversible trapping of the DNA-topoisomerase I covalent complex. Affinity labeling of the camptothecin binding site. *J. Biol. Chem.* **265**, 19287-19295.

Hoffman, R. M. (1999) Orthotopic metastatic mouse models for anti-cancer drug discovery and evaluation: a bridge to the clinic. *Invest. New Drugs* **17**, 343-359.

Itahaki, T., Itagaki, S., Sumi, Y., Hirano, T., Takemoto, I. and Iseki, K. (2005) Uptake of irinotecan metabolite SN-38 by the human intestinal cell line Caco-2. *Cancer Chemother. Pharmacol.* **55**, 420-424.

Iyer, L., Ramirez, J., Shepard, D. R., Bingham, C. M., Hosfield, D. K., Ratain, M. J. and Mayer, U. (2002) Biliary transport of irinotecan and metabolites in normal and P-glycoprotein-deficient mice. *Cancer Chemother. Pharmacol.* **49**, 336-341.

Kawato, Y., Aonuma, M., Hirota, Y., Kuga, H. and Sato, K. (1991) Intracellular roles of SN-38, a metabolite of the camptothecin derivative CPT-11, in the antitumor effect of CPT-11. *Cancer Res.* **51**, 4187-4191.

Khanna, R., Morton, C. L., Danks, M. K. and Potter, P. M. (2000) Proficient metabolism of irinotecan by a human intestinal carboxylesterase. *Cancer Res.* **60**, 4725-4728.

Kwak, E. Y., Shim, W. S., Chang, J. E., Chong, S., Kim, D. D., Chung, S. J. and Shim, C. K. (2012) Enhanced intracellular accumulation of a non-nucleoside anti-cancer agent via increased uptake of its valine ester prodrug through amino acid transporters. *Xenobiotica* [Epub ahead of print].

Mathijsen, R. H., van Alphen, R. J., Verweij, J., Loos, W. J., Nooter, K., Stoter, G. and Sparreboom, A. (2001) Clinical pharmacokinetics and metabolism of irinotecan (CPT-11). *Clin. Cancer Res.* **7**, 2152-2194.

Mulangi, R. and Srinivas, N. R. (2009) Clopidogrel: review of bioanalytical methods, pharmacokinetics/pharmacodynamics, and update on recent trends in drug-drug interaction studies. *Biomed. Chromatogr.* **23**, 26-41.

Rivory, L. P., Chaitelut, E., Canal, P., Mathieu-Boué, A. and Robert, J. (1994) Kinetics of the in vivo interconversion of the camptothecine and lactone forms of irinotecan (CPT-11) and of its metabolite SN-38 in patients. *Cancer Res.* **54**, 6330-6333.

Rothenberg, M. L. (2001) Irinotecan (CPT-11): recent developments and future directions–colorectal cancer and beyond. *Oncologist* **6**, 86-60.

Scott, D. O., Bindra, D. S., Sutton, S. C. and Stella, V. J. (1994) Urinary and biliary disposition of the lactone and carboxylic acid lactone forms of irinotecan (CPT-11) and of its metabolite SN-38 in rats. *Drug Metab. Dispos.* **22**, 438-442.

Sugawara, M., Huang, W., Fei, Y. J., Leibach, F. H., Ganapathy, V. and Ganapathy, M. E. (2000) Transport of valganciclovir, a ganciclovir prodrug, via peptide transporters PEPT1 and PEPT2. *J. Pharm. Sci.* **89**, 781-789.

Tallman, M. N., Ritter, J. K. and Smith, P. C. (2005) Differential rates of glucuronidation for 7-ethyl-10-hydroxy-camptothecin (SN-38) lactone and carboxylate in human and rat microsomes and recombiant UDP-glucuronosyltransferase isoforms. *Drug Metab. Dispos.* **33**, 977-983.

Umaphat, N. S., Ganapathy, V. and Ganapathy, M. E. (2004) Transport of amino acid esters and the amino-acid-based prodrug valganciclovir by the amino acid transporter ATB(0,+)*. *Pharm. Res.* **21**, 1303-1310.

Kwak et al. Investigation into the Efficacy of Val-SN-38