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Effect of SBE4-β-CD, a sulfobutyl ether β-cyclodextrin, on the stability and solubility of O^6-benzylguanine (NSC-637037) in aqueous solutions

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Abstract

The effect of SBE4-β-CD, a sulfobutyl ether derivative of β-cyclodextrin on the solubility and aqueous hydrolysis of the antitumor drug O^6-benzylguanine (BG) was studied. SBE4-β-CD is an apparently parenterally safe anionic β-cyclodextrin derivative with superior solubilizing properties in water. BG has poor aqueous solubility and undergoes rapid hydrolysis to the poorly water soluble guanine. The stability of a parenteral BG formulation was studied after storage at 25, 37 and 50°C. Compared to the intrinsic solubility of BG (0.14 mg/ml, 25°C), 0.05 M SBE4-β-CD enhanced its solubility to 2.9 mg/ml at 25°C and 3.9 mg/ml at 50°C. Solubility data yielded binding constants (K_b) of 565 M^{-1} at 25°C and 342 M^{-1} at 50°C. The solubility of guanine was only slightly enhanced by SBE4-β-CD. Hydrolysis kinetics of BG were studied at 50°C over a pH range of 1–9 and the maximum stability was observed at pH 8–8.5. In the presence of 0.05 M SBE4-β-CD, hydrolysis was about 9.5-times slower at pH 1, 14.6-times slower at pH 6 and 10-times slower at pH 8. The effect of SBE4-β-CD concentration was studied at pH 2.2 and 4.8 at 50°C. Hydrolysis rate constants decreased with increasing SBE4-β-CD concentrations. A non-linear regression analysis of this data yielded K_b values of 311 and 270 M^{-1} at pH 2.2 and 4.8, respectively. A formulation containing 2.5 mg/ml of BG and 0.05 M SBE4-β-CD in a pH 8 phosphate buffer was stored in ampoules at 25, 37 and 50°C. Guanine production in the samples was measured since its low solubility (2.5 μg/ml) imposed a limitation on the shelf life. Guanine levels exceeded its apparent solubility after 1–2 months of storage at 50°C. At 37°C guanine levels were only 1.6 μg/ml after 343 days of storage whereas those at 25°C were negligible and below the limit of quantitation (approx. 0.1 μg/ml). The greater stability at room temperature may be attributed to the higher K_b value observed and greater intrinsic stability of BG in the complex.

Keywords: O^6-Benzylguanine; Stability; Solubility; Anionic β-cyclodextrin derivative; SBE4-β-CD; Complexation; Parenteral formulation

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1. Introduction

The objective of the current study was to determine the effect of a sulfobutyl ether β-cyclodextrin (SBE4-β-CD), a proprietary β-cyclodextrin derivative (Rajewski, 1990; Stella and Rajewski, 1992) on the solubility and solution stability of O^6-Benzylguanine.

O^6-Benzylguanine (BG) is an effective antitumor drug enhancer. It is known to act by depleting the mammalian DNA repair protein O^6-alkylguanine-DNA alkyl transferase. Depleting tumor cells of this protein results in an enhancement of the activity of alkylating antitumor drugs which react with the O^6-position of guanine in DNA (Dolan et al., 1990, 1991). BG undergoes hydrolysis in aqueous media to form guanine and benzyl alcohol. The kinetics and mechanism of this process have been reported recently by Safadi et al. (1993). The rate-determining step involves an Sn1 mechanism with significant charge separation (Fig. 1). The pH-rate profile for BG hydrolysis was studied in the pH region between 1.0 and 5.2 and it was found to be quite unstable under acidic conditions. BG in addition to its hydrolytic degradation also presents a problem in developing injectable formulations due to its poor solubility in aqueous media. Further, the shelf life of such formulations may be severely limited by the poor aqueous solubility of the guanine formed during BG hydrolysis.

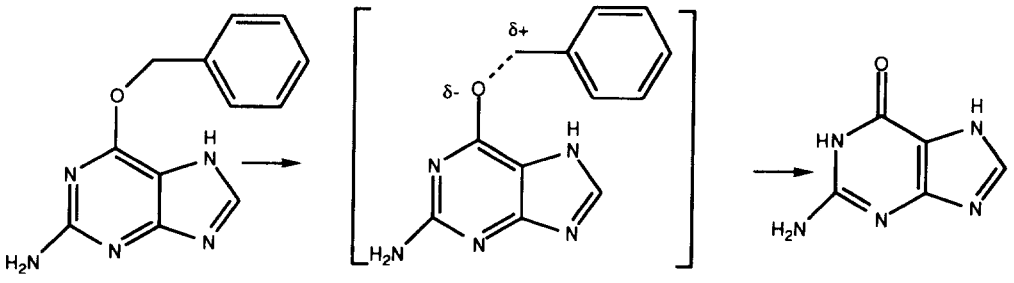
The effects of cyclodextrins on solubility and chemical stability of drug molecules have been extensively studied (Hirayama et al., 1986; Kikuchi et al., 1987; Si-Nang et al., 1989; Hora et al., 1992). However, the stabilization effects of SBE4-β-CD (Fig. 2) have not been reported previously. BG by virtue of its aromatic ring could be a suitable guest molecule for the cyclodextrin cavity. This interaction could be potentially beneficial for both solubilization and stabilization of the drug in order to achieve an acceptable parenteral formulation.

In the current study, the effects of SBE4-β-CD on the equilibrium solubilities of BG and guanine and on the pH-rate profiles of aqueous hydrolysis of BG were determined. Preliminary NMR studies were performed to determine the nature of the SBE4-β-CD-BG complex. The stability of a potentially acceptable parenteral formulation of BG was studied at various storage temperatures. Preliminary results on the formulation stability are reported.

2. Materials and methods

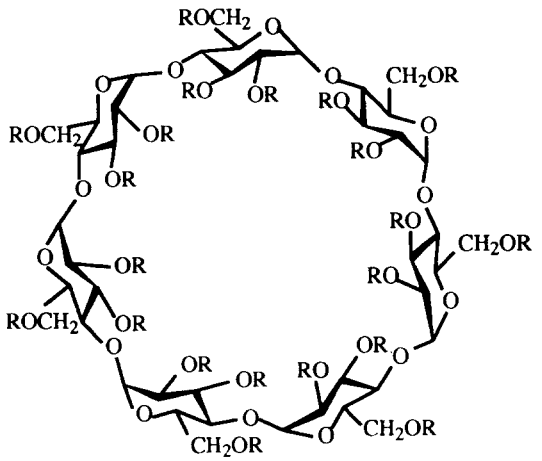
2.1. Materials

O^6-Benzylguanine (BG) was obtained from the National Cancer Institute (Bethesda, MD). SBE4-β-CD was provided by the Center for Drug Delivery Research (Lawrence, KS). Guanine was purchased from Sigma Chemical Co. (St. Louis,



O^6-BENZYLGUANINE GUANINE

Fig. 1. Reaction scheme for O^6-benzylguanine hydrolysis.



R = -$CH_2CH_2CH_2CH_2SO_3Na$ or -H
SBE4-β-CD
(average degree of sulfobutyl substitution is four)

Fig. 2. Schematic of the structure of SBE4-β-CD.

MO). All solvents were of HPLC grade and other reagents were of analytical grade.

2.2. Methods

2.2.1. Aqueous hydrolysis of BG

A BG stock solution was made in dimethyl sulfoxide (DMSO). Sample solutions of BG (42–150 μg/ml) containing 0.05 M SBE4-β-CD and appropriate buffers at the respective pH, as well as the corresponding control solutions without SBE4-β-CD, were incubated at 50°C in 10 ml volumetric flasks which were tightly closed and sealed with parafilm. The ionic strength of all the solutions was adjusted to 0.50 with sodium chloride (NB: the final ionic strength in solutions containing SBE4-β-CD was estimated to be approx. 0.5–0.6). Samples were withdrawn periodically and analyzed by HPLC. Samples with pH values lower than 6 were analyzed for benzylguanine disappearance. Samples at pH 6 and above being relatively more stable were analyzed for guanine production. The latter data were utilized to calculate hydrolysis rate constants using the initial rate approach.

2.2.2. HPLC analysis

BG analysis was carried out by the method of Safadi et al. (1993). A reverse phase C_{18} column (5 μm, 150 × 4.6 mm) was used. The mobile phase consisted of 50 mM phosphate buffer (pH 7), methanol and 1 mM *t*-butylammonium dihydrogen phosphate (50:50:1) at a flow rate of 1.5 ml/min. An ultraviolet detector was used at 280 nm. For guanine analysis, only the mobile phase in the above procedure was changed to 50 mM phosphate buffer (pH 7), methanol and 1 mM *t*-butylammonium dihydrogen phosphate (10:90:1) at a flow rate of 1.0 ml/min.

2.2.3. Equilibrium solubility studies

Excess amounts of BG were added to 0.1, 0.05, 0.025 and 0.0125 M solutions of SBE4-β-CD in water. The vials were tightly closed and agitated in 25°C and 50°C water baths for 24 h. Preliminary experiments showed that 24 h was sufficient to achieve equilibrium solubility with this compound. The samples were then centrifuged and the BG content in the supernatant was determined by HPLC. Guanine solubility in SBE4-β-CD solutions as well as the intrinsic solubilities of BG and guanine were determined in a similar fashion. The solubility data obtained was used to calculate binding constants for formation of the BG-SBE4-β-CD complex.

2.2.4. Effect of SBE4-β-CD concentration on BG hydrolysis

BG hydrolysis was monitored as described above at pH 2.2 and pH 4.8 in 0.015, 0.025 and 0.05 M SBE4-β-CD solutions containing 40–90 μg/ml of BG, at 50°C. The experimental data was mathematically treated to determine the binding constants for the formation of the BG-SBE4-β-CD complex and the rate constants for the hydrolysis of BG from the complex, at each pH.

2.2.5. NMR studies

All NMR spectra were recorded on a Bruker AM-500 operating at 500.14 M Hz for ^1H. A one-dimensional NMR spectrum of a saturated solution of BG in D_2O was used to make BG

proton assignments and a ROESY experiment was performed on a 0.08 M SBE4-β-CD containing about 4.5 mg/ml BG. ROESY conditions: 3500 Hz sweep width, 300 ms mixing time, 2K × 256 points, 80 scans/block, phase sensitive TPPI. Proton-proton distance information (interaction between specific protons on the BG molecule and SBE4-β-CD nucleus) was derived from cross-peaks in the ROESY contour plots.

2.3. Accelerated stability of an injectable BG formulation

A formulation was prepared containing 2.5 mg/ml of BG and 0.05 M SBE4-β-CD in a 0.05 M pH 8 phosphate buffer. Aliquots of 1 ml were sealed in glass ampoules and stored in constant temperature stability chambers maintained at 25, 37 and 50°C. Samples were periodically withdrawn and analyzed for their guanine content using the HPLC assay described above. Previous studies have shown that in this type of formulation, the rate of guanine production corresponds to the rate of BG loss. Sample concentrations were determined using guanine standard calibration curves.

3. Results and discussion

3.1. pH-rate profiles of BG hydrolysis

The effect of pH on the rates of BG hydrolysis at 50°C with and without SBE4-β-CD is shown in Fig. 3. The solid line for the pH-rate profile in absence of SBE4-β-CD represents rate constants calculated from the equation (Safadi et al., 1993) shown below.

$$k_{obs}(\min^{-1}) = k_H(H^+)(H^+/(H^+ + K_a)) + k'_H(H^+)(K_a/(H^+ + K_a)) \quad (1)$$

where $H^+/(H^+ + K_a)$ is the fraction of the drug in protonated form, $K_a/(H^+ + K_a)$ denotes the fraction of the drug in unprotonated form, k_H is the second-order rate constant for apparent attack of hydronium ion on the protonated form of the drug, and k'_H represents the second-order

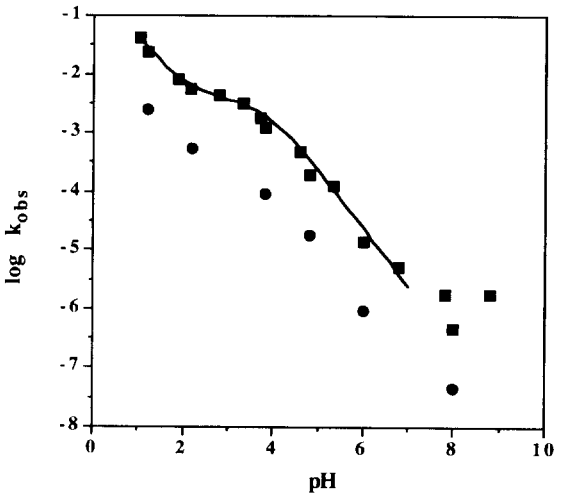


Fig. 3. pH-rate profile for the hydrolysis of O^6-benzylguanine at 50°C ($\mu = 0.5$). (a) Without SBE4-β-CD (B) and (■) With 0.05 M SBE4-β-CD (●). The solid line represents the theoretical profile without SBE4-β-CD, calculated from Eq. 1.

rate constant for the apparent attack of hydronium ion on the unprotonated form of the drug. The possibility of a k_o term or a term corresponding to the first-order rate constant for the apparent attack of water on the unprotonated form of drug can be envisaged at pH values greater than 7. However, the confidence in the existence of this term needs to be established by longer time studies. The calculated pH-rate profile in Fig. 3 was obtained by fitting Eq. 1 to the data using a nonlinear regression analysis program, Sigmaplot®. The rate and ionization constants (50°C, $\mu = 0.5$) calculated from the fit were as follows: $k_H = 0.39\ M^{-1}\ min^{-1}$, $k'_H = 24\ M^{-1}min^{-1}$, $K_a = 1.585 \times 10^{-4}$.

Rates of BG degradation in the presence of 0.05 M SBE4-β-CD were considerably slower than those in its absence, as shown in Table 1. The degradation rate in presence of 0.05 M SBE4-β-CD was approx. 9.5-times slower at pH 1, 14.6-times slower at pH 6 and 10.6-times slower at pH 8.

3.2. Equilibrium solubility and binding constant determination

BG solubility was significantly enhanced by the use of SBE4-β-CD (Table 2) and was seen to

Table 1
Rate constants for O^6-benzylguanine hydrolysis in the presence and absence of 0.05 M SBE4-β-CD at 50°C

pH	k_{obs} (min^{-1})	$k_{obs/SBE}$ (min^{-1})	Ratio = $k_{obs}:k_{obs/SBE}$
1.2	2.36×10^{-2}	2.51×10^{-3}	9.4
2.2	5.46×10^{-3}	5.42×10^{-4}	10.1
3.8	1.21×10^{-3}	9.50×10^{-5}	12.7
4.8	1.92×10^{-4}	1.79×10^{-5}	10.7
6.0	1.39×10^{-5}	9.52×10^{-7}	14.6
8.0	4.78×10^{-7}	4.49×10^{-8}	10.6

depend linearly on the SBE4-β-CD concentration up to 0.1 M. There was an approx. 21-fold increase in the solubility of BG at 25°C to 2.9 mg/ml in the presence of 0.05M SBE4-β-CD, in comparison with its intrinsic solubility of 0.14 mg/ml. The equilibrium solubility of BG at 50°C in 0.05 M SBE4-β-CD was determined to be 3.9 mg/ml. The equilibrium solubility data was used to determine the binding constants (K_b) for a 1:1 complex formation between BG and SBE4-β-CD. K_b values of 565 M^{-1} at 25°C and 342 M^{-1} at 50°C were obtained.

Guanine produced as a result of BG hydrolysis has a very low aqueous solubility (2.5 μg/ml, water, 25°C) and hence guanine levels attained could be a potential limiting factor in the shelf life of a BG parenteral formulation. Since the target concentration of BG in the formulation was 2.5 mg/ml, guanine solubilities were determined in the presence of 2.5 mg/ml BG dissolved in 0.05 and 0.1 M SBE4-β-CD. While guanine solubility appeared to increase with an increase in SBE4-β-CD concentrations the increase was relatively small. The low solubility of guanine in SBE4-β-CD in the presence and absence of BG presumably stems from the low interaction constant, a K_b value of 3.5 M^{-1} being estimated. This low K_b, considerably smaller than that for the BG-SBE4-β-CD complex, also suggests that the benzyl moiety of BG is incorporated into the β-cyclodextrin cavity. This was confirmed by NMR experiments where a ROESY experiment showed NOEs between all the aromatic benzylic protons, especially those in the *meta* and *para* position, to protons between 4.05 and 3.858 in SBE4-β-CD. There was no apparent NOEs between the benzylic methylene protons and any protons of SBE4-β-CD suggesting that the benzyl group was only partially buried in the cyclodextrin nucleus. There were no NOEs between the protons on the guanine nucleus part of BG with SBE4-β-CD protons confirming that the guanine portion of the molecule did not participate in the interaction.

3.3. Effect of SBE4-β-CD concentration on BG hydrolysis

The effect of SBE4-β-CD on the degradation of BG is shown in Fig. 4 and in Table 3. The hydrolysis rates decreased hyperbolically with increasing cyclodextrin concentration. The BG is better stabilized by SBE4-β-CD at pH 4.8 than at pH 2.2. This observation is in agreement with the pH-rate profile described earlier.

Scheme 1 represents the complexation of BG with SBE4-β-CD and degradation of both un-

Table 2
Equilibrium solubilities of O^6-benzylguanine and guanine in SBE4-β-CD solutions

SBE4-β-CD concentration (M)	O^6-Benzylguanine solubility (mg/ml) at 25°C	O^6-Benzylguanine solubility (mg/ml) at 50°C	Guanine solubility (mg/ml) ($\times10^3$) at 25°C
0.1	6.0	–	4.4
0.05	2.9	3.9	3.6
0.025	1.5	2.2	2.9
0.0125	0.8	1.0	2.7
0.1 (with 2.5 mg/ml BG)	–	–	3.7
0.05 (with 2.5 mg/ml BG)	–	–	3.6
0 (intrinsic solubility)	0.14	0.32	2.5

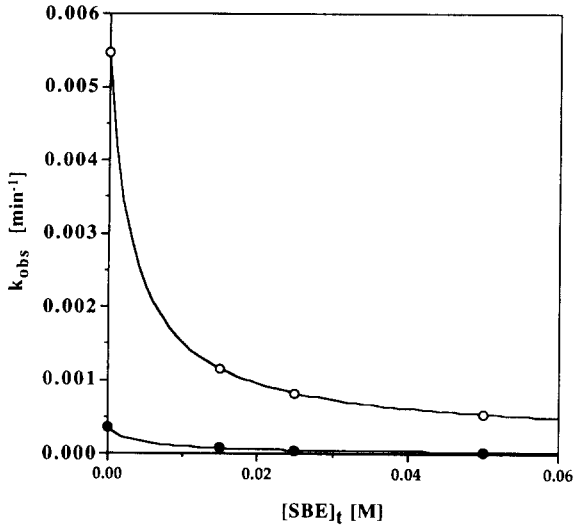
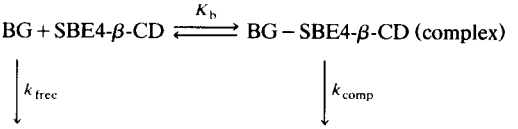


Fig. 4. Effect of SBE4-β-CD on the observed rate constants for the hydrolysis of O^6-benzylguanine at 50°C at pH 2.2 (○) and at pH 4.8 (●). The solid lines at each pH represent the theoretical rate constants calculated from fits to Eq. 2.

complexed and complexed drug. The observed rate constant (k_{obs}) for the hydrolysis of BG depends on SBE4-β-CD concentration according to the following equation:

$$k_{obs} = \frac{k_{free} + (K_b k_{comp}[SBE]_t)}{1 + K_b[SBE]_t} \quad (2)$$

Based on Scheme 1 showing 1:1 complexation, K_b is the binding constant for the complexation, k_{free} denotes the pseudo first-order rate constant for the degradation of free BG, k_{comp} is the pseudo first-order rate constant for the degradation of BG from its inclusion complex, and $[SBE]_t$ the total concentration of SBE4-β-CD. Non-linear regression analysis was performed using Sigmaplot® to fit the data to Eq. 2. Identical



Scheme 1.

results were obtained when the program Minsq® (Micromath, UT) was used. The rate constants for degradation of BG from its inclusion complex at pH 2.2 and 4.8 were found to be 2.2×10^{-4} and 1.6×10^{-6} min^{-1}, respectively. This corresponds to a hydrolysis rate constant lower by a factor of 24 at pH 2.2 and by a factor of 219 at pH 4.8, relative to the rates of hydrolysis of free BG under the same conditions. The binding constants were determined to be 311 M^{-1} at pH 2.2 and 270 M^{-1} at pH 4.8. The hydrolysis of BG itself was expected to be slower at the higher pH based on the results described earlier. This could be the basis of the approx. 10-fold greater reduction in hydrolysis of complexed BG, at pH 4.8 than at pH 2.2, in spite of the similar and low binding constants observed at both pH values.

The acid-catalyzed degradation of BG has been shown to proceed through an Sn1 mechanism with significant charge separation in the rate-determining step (Safadi et al., 1993; see Fig. 1). If the benzyl ether linkage were to be partially buried in the relatively hydrophobic cavity of SBE4-β-CD, build-up of the carbocation character on the benzylic carbon would not be favored. For example, Sn1 reactions are not favored in low dielectric environments.

3.4. Effect of temperature

The preliminary formulation was made at pH 8 to provide conditions of maximum stability of

Table 3
Rate and stability constants for O^6-benzylguanine and the SBE4-β-CD-O^6-benzylguanine complex at 50°C in KCl-HCl buffer pH 2.2 and acetate buffer pH 4.8

	k_{free} (min^{-1})	$k_{complex}$ (min^{-1})	$k_{free}/k_{complex}$	K_b (M^{-1})
pH 2.2 benzylguanine	5.5×10^{-3}	–	–	–
pH 2.2 benzylguanine-SBE4-β-CD	–	2.2×10^{-4}	24	311
pH 4.8 benzylguanine	3.5×10^{-4}	–	–	–
pH 4.8 benzylguanine-SBE4-β-CD	–	1.6×10^{-6}	219	270

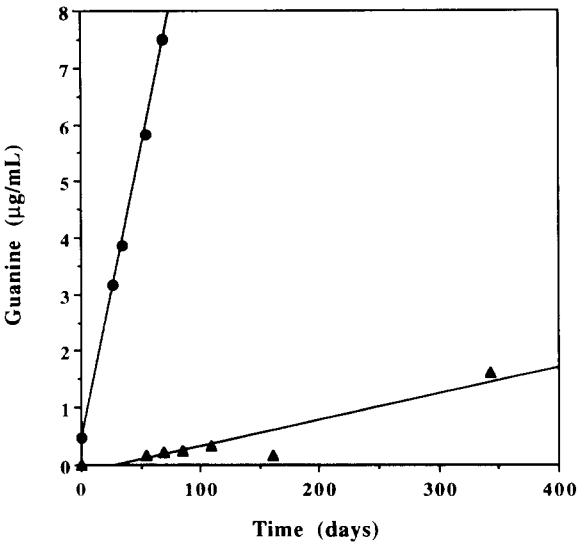


Fig. 5. Formation of guanine in a formulation containing 2.5 mg/ml of O^6-benzylguanine and 0.05 M SBE4-β-CD in a 0.05 M pH 8 phosphate buffer, after storage at 37°C (▲) and 50°C (●). At 25°C no significant production of guanine was seen.

BG as indicated by the pH-rate profiles for BG hydrolysis. Due to the high stability of BG, initial rate estimates based on guanine concentrations measured in the samples were used to determine stability. Also, since the guanine concentrations are expected to be an important factor in determining the formulation shelf life, the results (Fig. 5) provided a good estimate of this effect. The samples stored at 50°C were most unstable and guanine concentrations exceeded its solubility within 1–2 months. At 37°C guanine concentrations were about 1.6 μg/ml after storage for 343 days. There seems to be a disproportionate effect of temperature on the stability of BG in the presence of SBE4-β-CD. At 25°C guanine concentrations were negligible (< 0.1 μg/ml) even after 343 days. The greater stability observed at 25°C may be due to the higher binding constant and hence a greater intrinsic stability of the BG in the complex relative to that at 50°C.

In conclusion, SBE4-β-CD was highly effective in improving the aqueous solubility and chemical stability of BG. Solubility and binding constant data indicated that the benzyl moiety of BG was critical for the formation of the complex. While SBE4-β-CD did not appreciably increase the solubility of guanine, the increased stability of BG allowed an increase in the shelf life of a parenteral formulation of the drug.

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