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RESEARCH PAPER

Physicochemical Properties of CWJ-a-5, a New Antitumor 3-Arylisoquinoline Derivative

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ABSTRACT

The compound CWJ-a-5 [1-(4-methylpiperazinyl)-3-phenylisoquinoline hydrochloride] is a novel 3-arylisoquinoline derivative which has exhibited potent antitumor activity. As part of an effort to develop a useful formulation for clinical evaluation of this compound, the aqueous stability of CWJ-a-5 as a function of pH, ionic strength, and temperature, as well as its various physicochemical properties, have been examined. The pK_a *value obtained by potentiometric titration in methanol–water mixtures was 3.61, at 25°C. The aqueous solubility and the apparent partition coefficient of CWJ-a-5 over the pH range 2.08–9.88 were consistent with those expected of a weak acid of similar* pK_a *value. The degradation of CWJ-a-5 was found to follow apparent first-order kinetics. The pH–rate profiles generated at 80°C were accounted for by acid-catalyzed degradation at low pH and base-catalyzed degradation at high pH. The activation energy was determined as 22.12 kcal/mol for the degradation of CWJ-a-5 in a pH 2.92 solution with a constant ionic strength of 0.2. Increasing the ionic strength up to 0.9 led to a higher degradation rate constant at pH 2.92.*

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However, CWJ-a-5 was very stable even in a pH 2.92 solution, and its shelf-life was calculated to be 2.03 years at 25°C from the Arrhenius plot.

Key Words: *CWJ-a-5; Antitumor; Isoquinolone; Physicochemical*

INTRODUCTION

The clinical success of fagaridine,[1,2] which is a natural phenolic benzo[c]phenanthridine alkaloid, has drawn considerable attention towards the synthesis and biological evaluation of its related compounds.[3,4] However, only limited structure–activity relationship studies are found because most of the synthetic procedures for the preparation of benzo[c]phenanthridines face certain problems in producing a diverse series of substituted analogs.[5] During the course of research with these phenolic benzo[c]phenanthridines, a strong antitumor agent 7,8-dimethoxy-2-methyl-3-(4,5-methylenedioxy-2-vinylphenyl)-isoquinoline-1(2*H*)-one (IC_{50} = 0.2 nM: SK-MEL-2) was discovered.[6]

With this new arylisoquinoline derivative as the lead compound, a structure–activity relationship study was conducted together with a systemic pharmacophore study. In order to enhance the water solubility, the amide group was converted to an *N*-methyl-piperazinyl group. Among these analogs tested, 1-(4-methylpiperazinyl)-3-phenylisoquinoline (CWJ-a-5, Fig. 1) displayed a potent and broad antitumor activity against five human cell lines, A 549 (lung), SKOV-3 (ovarian), SK-MEL-2 (melanoma), XF 498 (CNS), and HCT 15 (colon) in the in vitro cytotoxicity assay.[7–9] Moreover, CWJ-a-5, which showed comparable activity as fagaridine in vitro, showed 160 T/C% in an in vivo assay using C57BL/6 mice inoculated with B16F10 melanoma.[10] Not only does CWJ-a-5 exhibit potent antitumor activity, it was also proven to have low toxicity. In the acute toxicity test after intraperitoneal administration to SPF (specific pyrogen-free) mice, the LD_{50} was 701.54 mg/kg.[11] In vitro chromosome aberration test using newborn CHL (Chinese hamster lung) tissue and Ames test results showed CWJ-a-5 to be a good candidate for clinical studies.

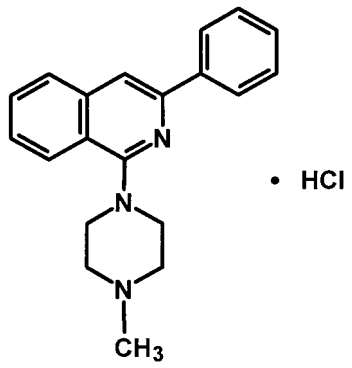

Figure 1. Chemical structure of CWJ-a-5.

To develop CWJ-a-5 into a clinical antitumor agent, its physicochemical properties for an optimum formulation must be investigated. A pharmacokinetics study in rats has been reported previously, where CWJ-a-5 was shown to be water-soluble in an acidic condition and to have a large volume of distribution. Moreover, the oral bioavailability was 50%, which indicated the possibility of it being developed into an oral dosage form.[12] In continuation of the preformulation study, fundamental physicochemical properties such as the apparent dissociation constant, the solubility, the apparent partition coefficient, and the aqueous stability are described herein.

MATERIALS AND METHODS

Materials

The hydrochloride salt of CWJ-a-5 was synthesized in the Medicinal Chemistry Laboratory of Chonnam National University (Kwangju, South Korea) as described previously.[7,13,14] Methanol of HPLC grade was purchased from Merck (Darmstadt, Germany). Solvents for HPLC were filtered through a 0.22-μm filter and thoroughly degassed in an ultrasonic bath before use. All other reagents were of analytical grade and used without further purification.

Dissociation Constant Determination

Due to the low solubility of CWJ-a-5 in basic aqueous media, its pK_a was determined by the

potentiometric titration of a series of semi-aqueous CWJ-a-5 solutions of known concentration and 29.84–49.12% (w/w) of methanol, as reported in the literature.[15] The temperature was set at 25°C. Solutions of CWJ-a-5 equivalent to 10^{-3} M were titrated with 0.01 M sodium hydroxide, with parallel addition of methanol to maintain the constant methanol/water composition. The estimated pK_a corresponding to a zero value of methanol was obtained from the Yasuda–Shedlovisky plot that uses p_sK (apparent pK_a) + log[H_2O] vs. $100/D$, wherein D is the dielectric constant.[16]

Solubility Measurements

The solubilities of CWJ-a-5, its free base form, and its oxalate salt form in ethanol, methanol, and distilled water were measured at 25°C. An excess of each compound was added to each solvent and mixed by vortexing. The solution was mechanically shaken for 72 hr at 25°C to allow equilibration. The saturated solutions were then filtered through Minisart RC 4 filters (0.45 μm, Sartorius, Germany). Concentrations of the compounds were analyzed by high-performance liquid chromatography (HPLC) after appropriate dilution. The solubilities of CWJ-a-5 in aqueous buffer solutions at various pHs were also determined by the same procedure. The aqueous buffer solutions used in this study were prepared by USP XXIV, and the ionic strength was adjusted to 0.2 with sodium chloride.

Apparent Partition Coefficient Measurements

The partition coefficient was determined in triplicate according to the method of Dearden and Bresnen.[17] The distilled water or various aqueous buffer solutions were mutually saturated with 1-octanol by gentle mechanical stirring for 12 hr, after which each phase was separated. Methanolic solution of CWJ-a-5 (10 mg/mL) was placed in a glass tube (30 μL), and 3.0 mL of each saturated solvent was added to the tube after completely evaporating the methanol. After shaking of the stoppered tube for 24 hr at 20 inversions/min, the phases were separated by centrifugation at 3000 rpm for 20 min. The concentration of CWJ-a-5 in each phase was determined by HPLC.

Solution Stability Studies

A stock solution of CWJ-a-5 (2 mg/mL) was prepared in double distilled water. All buffer solutions were 0.2 M and adjusted to 0.2 ionic strength with sodium chloride. Aliquots (0.25 mL) were taken from the stock solution and diluted to 25 mL with the appropriate buffer to make a final concentration of 20 μg/mL. The solution aliquots were withdrawn and sealed in 5 mL amber glass ampules (Type I, Wheaton Scientific, Millville, NJ). For the kinetic studies, these samples were placed in a dark mechanical convection oven (EYELA NDO-600ND, Tokyo Rikakikai Co. Ltd., Japan) maintained at 80°C for up to 85 days. At appropriate time intervals, samples were removed from the oven and equilibrated to room temperature prior to assay. The pH values were checked (Microprocessor pH meter HI 9321, HANNA Instruments, Portugal) for each sample to detect any significant change of pH at each designated time. Concentrations of CWJ-a-5 were determined in triplicate by HPLC. The initial concentration was considered as 100%, and the remaining concentrations were expressed as a percentage of the initial.

An accelerated stability study was conducted at temperatures of 80°C, 90°C, 100°C, and 110°C, using CWJ-a-5 (20 μg/mL) in 0.2 M hydrochloric acid buffer solutions (pH 2.92) with constant ionic strength ($\mu = 0.2$). Various ionic strengths ($\mu = 0.2$ to 0.9) of CWJ-a-5 in 0.2 M hydrochloric acid buffer solutions (pH 2.92) were used to study the effect of ionic strength on the aqueous stability of CWJ-a-5 (20 μg/mL) at 80°C.

HPLC Assay

The concentration of CWJ-a-5 was determined using an HPLC system equipped with a binary pump system (Gilson Model 305 and 306) and automatic injector (Gilson Model 234). A Merck C18 LiChroCART® 250-4 column (5 μm, 250 × 4 mm^2, Merck, Darmstadt, Germany) was used for analysis at ambient temperature. The mobile phase consisted of methanol, 10 mM ammonium acetate, and 25% ammonia water in a volume ratio 89.8:9.7:0.5 (its final pH was 10.0). The variable wavelength ultraviolet detector (Gilson Model 118) was set at 254 nm. Injections of 20 μL were made for all solutions to be analyzed. With a flow rate of

1.0 mL/min, the retention time for CWJ-a-5 was 9.3 min, and the detection limit was less than 50 ng/mL.

RESULTS AND DISCUSSION

Apparent Dissociation Constant

Figure 2 shows the Yasuda–Shedlovisky plot of CWJ-a-5 in various weight fractions of methanol/water mixture at 25°C. The pK_a value of CWJ-a-5 in aqueous solution was estimated by extrapolating the regression line and determining the $p_sK + \log[H_2O]$ value at a zero value of methanol. The pK_a value of CWJ-a-5 obtained by this model was 3.61, corresponding to the deprotonation of the piperazine nitrogen. The percentage of protonated or non-protonated forms of CWJ-a-5 in each specific pH solution can be obtained from this information, i.e., when the pH is higher than 5.61, more than 99% of CWJ-a-5 is expected to be in the non-protonated form.

Solubility and Apparent Partition Coefficient

Table 1 shows the solubility of CWJ-a-5 and its free base form in several solvents at 25°C. All forms of the compound were most soluble in methanol. The free base form was rarely soluble in water, but very soluble in ethanol and methanol. The hydrochloride salt (CWJ-a-5) showed relatively high solubility in both water and the alcohols. Figure 3 shows the solubility of CWJ-a-5 in various pH buffer solutions when equilibrated for 72 hr at 25°C: CWJ-a-5 was most soluble in acidic medium, but rarely soluble in basic medium.

The apparent partition coefficients ($\log P$) of CWJ-a-5 between 1-octanol and buffer solutions are shown in Fig. 3. Here we see that CWJ-a-5 is lipophilic with 1-octanol/water partition coefficient value of 2.64 (± 0.02). A higher $\log P$ value in a basic medium than in an acidic medium suggests that the higher fraction of CWJ-a-5 exists as a free base form in basic solution while as a protonated form in acidic solution. The aqueous solubility and apparent partition coefficient of CWJ-a-5 over the pH range 2.08–9.88 were consistent with those expected of a weak acid with a similar pK_a value.

Table 1

Solubility of CWJ-a-5 Free Base and Salts in Various Solvents at 25°C

	Solubility (mg/mL)		
Salt	Water	Ethanol	Methanol
Free base	$9.54\ (1.85) \times 10^{-3}$	≫400	≫400
Oxalate salt	1.53 (0.07)	0.79 (0.06)	9.18 (0.19)
HCl salt (CWJ-a-5)	69.75 (8.05)	9.81 (0.52)	189.23 (7.3)

Each value represents the mean (±SD) of three experiments.

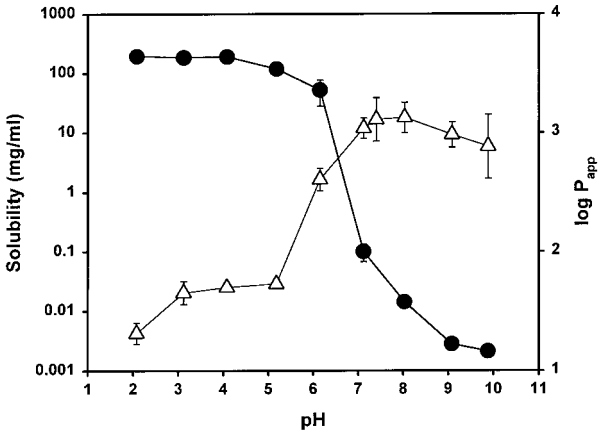

Figure 3. Apparent 1-octanol/water partition coefficient (Δ) and solubility (●) of CWJ-a-5 at 25°C in various buffer solutions, where the ionic strength of the buffer was adjusted to 0.2.

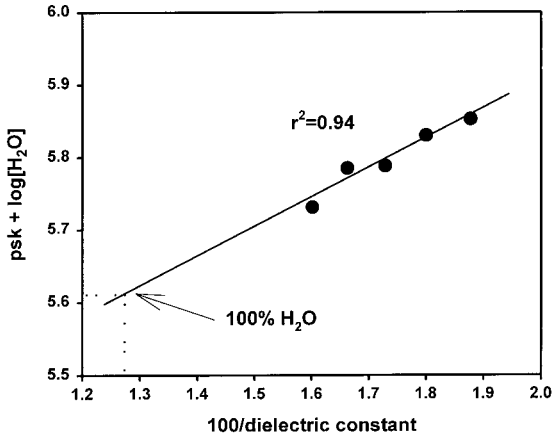

Figure 2. Yasuda–Shedlovisky plot for CWJ-a-5 in methanol/water mixtures.

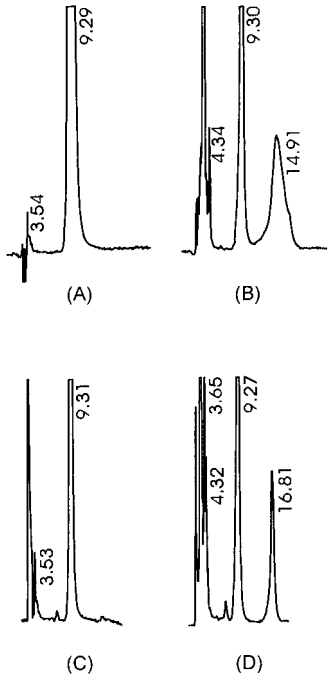

Figure 4. The HPLC chromatograms of CWJ-a-5 (20 μg/mL) in (A) pH 2.92 when stored at 80°C for 0 hr, (B) pH 2.92 when stored at 80°C for 33 hr, (C) pH 7.01 when stored at 80°C for 0 day, and (D) pH 7.01 when stored at 80°C for 21 days.

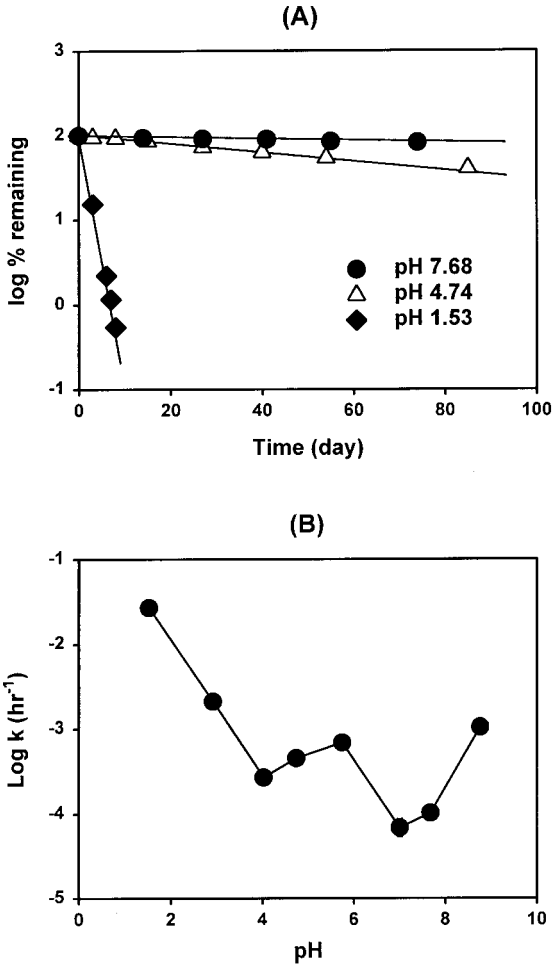

Figure 5. (A) Apparent first-order degradation of CWJ-a-5 (20 μg/mL) in pH 1.53 (◆), pH 4.74 (Δ), and pH 7.68 (●) buffer solutions (0.2 M, $\mu = 0.2$) at 80°C. (B) pH–rate profile of CWJ-a-5 in various buffer solutions (0.2 M, $\mu = 0.2$) at 80°C.

Stability Studies

The stability-indicating nature of the HPLC assay was depicted by the chromatograms (Fig. 4) where samples of CWJ-a-5 (20 μg/mL) in pH 2.92 and 7.01 buffer solutions were degraded at 80°C. When these solutions were stored at 80°C, the CWJ-a-5 peak decreased, producing unidentified degradation products. However, CWJ-a-5 was appropriately separated from the other substances in the samples, which made it possible to calculate the degradation kinetics. Different HPLC chromatograms of CWJ-a-5 after degradation in pH 2.92 and 7.01 solutions indicate that the degradation mechanism of this compound could be different in each buffer solution.

As shown in Fig. 5A, the degradation of CWJ-a-5 in various buffer solutions follows typical first-order kinetics. The degradation rate constants were calculated by the regression analysis method, and plotted as a function of pH of solutions with constant ionic strength of 0.2 at 80°C (Fig. 5B). At pH lower than 4.0, the degradation of CWJ-a-5 could be described primarily by the catalytic effect of hydrogen ion on the protonated form of CWJ-a-5. At pH 4.0–7.0, its degradation was primarily due to the catalysis of hydrogen ion or water on the non-protonated form of CWJ-a-5. At pH higher than 7.0, the catalysis of hydroxyl ion or water seemed to become predominant.

Although the Debye–Hückel equation is well obeyed in solutions with ionic strengths up to 0.01, pharmaceutical investigations are most often conducted at an ionic strength range of >0.01. Thus, the following modified Debye–Hückel equa-

tion is more commonly used in pharmaceutical formulations:[18]

$$\log k = \alpha + 2QZ_AZ_B[\mu^{1/2}/(1+\mu^{1/2})]$$

where k is the reaction constant, α is the reaction rate constant at infinite dilution of given solvent, $2Q$ is the constant for a given solvent and temperature, Z_AZ_B is the charge on reactant A and B, and μ is the ionic strength of the solution.

In this study, it was necessary to keep the concentration of buffering agents high so that a constant pH environment could be maintained. Due to the high ionic strength created by the buffer (0.2 M) itself, a higher ionic strength range was used ($\mu = 0.2$ to 0.9). Figure 6 shows the salt effect on the degradation of CWJ-a-5 in a pH 2.92 solution by plotting $\log k$ vs. $\mu^{1/2}/(1+\mu^{1/2})$. The result for the pH 2.92 solution followed the modified Debye–Hückel equation well up to an ionic strength of 0.9. The $2QZ_AZ_B$ value obtained from the slope of the plot was 1.75. Since Q is a function of temperature, a value of 1.145 for $2Q$ in aqueous solution at 80°C was cited from the literature.[19] The observed Z_AZ_B value was calculated to be 0.76. Under the assumption that two molecules collide at once, the theoretical value of Z_AZ_B should be equal to 1.0 at pH 2.92 buffer because of the specific acid catalysis on the protonated form of CWJ-a-5. The variation between the observed and theoretical values may be due to the secondary salt effect from the high ionic strength of the solutions.

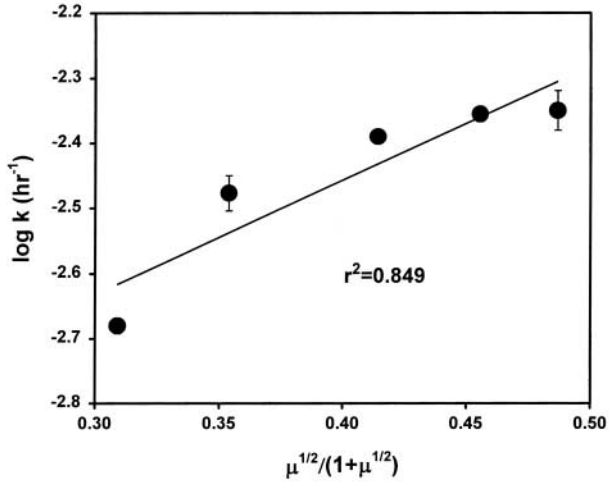

Figure 6. Salt effect on the degradation of CWJ-a-5 (20 μg/mL) in pH 2.92 buffer solution at 80°C.

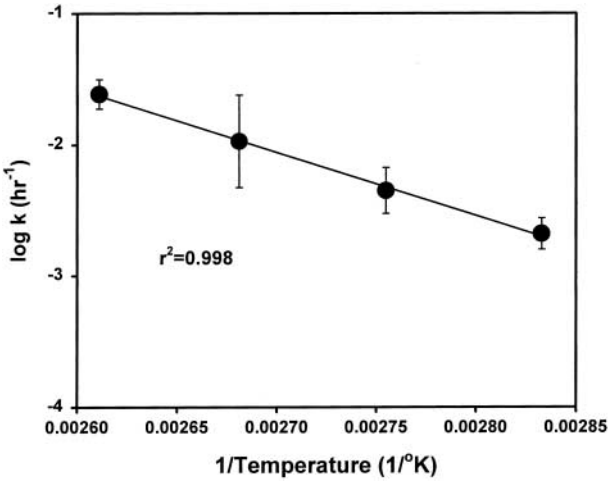

Figure 7. Temperature dependence of the degradation rate of CWJ-a-5 (20 μg/mL) in pH 2.92 (0.2 M, $\mu = 0.2$).

The degradation rate of CWJ-a-5 (20 μg/mL) as a function of temperature (80–110°C) in pH 2.92 buffer at constant ionic strength of 0.2 was demonstrated by plotting the logarithm of the degradation rate constant vs. 1/temperature as shown in Fig. 7. The Arrhenius plot was linear, indicating that the degradation mechanism was not altered with a change in temperature inside the temperature range of study. The energy of activation in this solution was calculated from the slope and was determined to be 22.12 kcal/mol for pH 2.92 buffer solution. Then, the degradation rate constant of CWJ-a-5 at 25°C was estimated to be $5.93 \times 10^{-6}\,hr^{-1}$ ($t_{90} \sim 2.03$ year) in a pH 2.92 buffer solution.

In conclusion, CWJ-a-5 was proven to be a stable water-soluble drug candidate. Its stability in acidic conditions and lipophilic nature in physiological pH could explain the high oral bioavailability and large volume of distribution.

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