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Improved Oral Absorption of a Poorly Water-Soluble Drug, HO-221, by Wet-Bead Milling Producing Particles in Submicron Region

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***N*-[[[4-(5-Bromo-2-pyrimidinyloxy)-3-chlorophenyl]amino]carbonyl]-2-nitrobenzamide (HO-221) is being developed as an anticancer agent with a novel mode of action. HO-221 shows poor oral absorption and is only slightly soluble in water (0.055 μg/ml at 37 °C). In this study, it was shown that the reduction in particle size of HO-221 to the submicron region (0.453 μm, mean by volume) could be achieved by a wet milling in a decaglycerin monolaurate aqueous solution with small glass beads. The wet milling suspension obtained showed improved dissolution rate and oral absorption in rats. A solid dosage form be could also made from that suspension with addition of sucrose palmitate which prevented aggregation caused by the hydrophobic interaction. The solid dosage form thus obtained showed twice as much oral absorption in dogs as the preparation made by dry milling.**

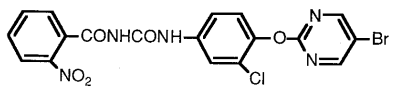
Keywords oral absorption; poorly water-soluble drug; micronization; submicron particles; wet-bead milling; dosage form

A variety of phenylbenzoylurea derivatives, which were once synthesized in the search of potential candidates of agricaltural chemicals,[1] were studied by the authors to find favorable anticancer drugs possessing a novel mode of action. After the screening tests, *N*-[[[4-(5-bromo-2-pyrimidinyloxy)-3-chlorophenyl]amino]carbonyl]-2-nitrobenzamide (signified hereafter as HO-221, Fig. 1) was found to act as a strong inhibitor of DNA polymerase alpha activity and selected as a candidate compound to be developed.[2]

However, HO-221 exhibits extremely poor solubility in water: as low as 0.055 μg/ml (See Experimental section). This inherent property has caused it to be viewed as inappropriate for use in medicinal drugs, because of predictable insufficient gastrointestinal absorption as well as the difficulty in formulating injection preparations. Nonetheless, it seems to us to be a challenge to tame such a difficult compound and to learn its antitumor activity.

Various methods of increasing the bioavailability of poorly water-soluble drugs have been reported elsewhere: solid dispersion,[3] solubilization into organic solvents,[4] micronization[5] and others. Our particular interest was in the technology of micronization.

Particle size reduction of a powdered drug by micronization may increase the surface area of the drug particles enhancing the dissolution rate of the active ingredient and hence its bioavailability.[6] In the pharmaceutical field, a dry milling process using an atomizer or a jet mill is popular.[7] This process has been reported to produce fine particles as small as 3 μm in diameter.[8] In chemical fields where paints (titanium oxide), magnetic (iron oxide) and fine ceramics[9] are used, a wet-bead milling process has been industrially applied to effect to a submicron region, typically 0.1—1 μm. There are examples of the wet milling of organic compounds to a submicron region in the area of agricultural chemicals.[10] However, there has been no precedent, to our knowledge, for the application of wet-bead milling to the pharmaceutical field.



$C_{18}H_{11}BrClN_5O_5$:M.W.492.67

Fig. 1. Chemical Structure of HO-221

The purposes of the present study were to establish a method for micronizing HO-221 to the submicron region using a wet-bead mill in a suspension form, to make a solid form from the suspension, and to determine the effect of this particle size reduction on its oral absorption in animals.

Experimental

Chemicals HO-221 was synthesized by Ishihara Sangyo Kaisha, Ltd., Japan. It occurs as a needle-shaped crystal with a melting point ranging 222.5—228.5 °C. Solubility in water and partition coefficient (octanol: water) determined by an HPLC method[11] were 0.055 μg/ml at 37 °C and 2.46 ($\log P_{oct}$), respectively. This powder was ground, if necessary, with a jet mill (TJ60, Tabo Kogyo K. K., Japan) in the usual manner. Polyoxyethylene hydrogenated castor oil 60 (HCO-60) and decaglycerin monolaurate were obtained from Nikko Chemical Company, Ltd., Japan. Sucrose palmitate (hydrophilic lipophilic balance; approx. 16) was obtained from Mitsubishi Kasei Food Corp., Japan. All other chemicals were of analytical reagent grade and were used as received.

Wet-Bead Milling A wet-bead milling was carried out using a Dyno-Mill, type KDL (Willy A. Bachofen AG Maschinenfabrik, Switzerland) with lead-free glass beads. The suspension of 30% (w/v) of HO-221 in 5% (w/v) of decaglycerin monolaurate aqueous solution was roughly mixed by a homogenizer (Biotron, Biotrona, Switzerland), and poured into a 0.6 l grinding-glass container filled with glass beads of 0.25—0.5 mm diameter. Decaglycerin monolaurate was added to prevent particle flocculation. Milling was carried out at 3000 rpm with cooling at 20 °C.

Scanning Electron Micrography Shapes and sizes of HO-221 particles following the milling were observed using a scanning electron microscope (H600/6010, Hitachi Instrument Engineering Co., Ltd., Japan).

Particle Size Measurements There is no appropriate particle size analyzer to measure all the broadly varying particle sizes simultaneously, from under one micron to more than 100 μm. Therefore, two kinds of analyzer were required: one for wet-bead milling is a submicron particle size instrument employing photon correlation spectroscopy, Autosizer model IIc (Malvern Instruments, England) capable of measuring 0.01—3 μm,[12] and the other is a laser-based time of transition analysis system, CIS-1 (Galai Production Ltd., Israel) which covers the range of 0.5—150 μm.[13] Appropriate amounts of test preparations were suspended in an aqueous solution of 0.005% (w/v) HCO-60 before measurement.

Mean particle size was expressed as average by volume.

Dissolution Studies Dissolution experiments were performed using the JP paddle method.[14] Low solubility of HO-221 in an aqueous medium requires the addition of a detergent to the medium to solubilize the test material. An aqueous solution of 1% (w/v) HCO-60 was selected as the testing medium to determine dissolution. Test preparations equivalent to 2 mg of HO-221 were added to 900 ml of the dissolution medium which was maintained at a temperature of 37±0.1 °C. As the solubility of HO-221 in 1% (w/v) HCO-60 is 7.678 μg/ml at 37 °C, the amount of HO-221 added was within the range of sink conditions. The stirring speed used was 50 rpm. Samples (5 ml) were withdrawn at 10, 30, 60 and 120 min and centrifuged at 15000×*g* for 10 min. Supernatants were analyzed by the HPLC method described in the section on assay of HO-221 (*vide infra*). After each sampling, 5 ml of medium was added to the testing medium as a replacement. From the concentrations of HO-221 thus determined, the cumulative percentage was calculated.

Oral Absorption in Rats or Dogs Wistar strain male rats weighing about 200 g and male beagle dogs weighing about 10 kg were used. Preparations were suspended in water and administered orally through a sonde at a dose of 25 mg/kg. Blood samples (0.3 ml for rats and 3 ml for dogs) were obtained from the jugular vein or the leg vein, respectively, with a preheparinized syringe and temporarily stored on ice. Whole blood was centrifuged at 5000×*g* for 10 min, and plasma was collected for analysis. To 100 μl of plasma, an internal standard solution which contained 2.5 μg/ml of diphenyl in acetonitrile was added followed by vortex mixing and centrifugation at 5000×*g* for 10 min, resulting in deproteinization of the plasma. Supernatant (50—80 μl) was injected into the analytical column described below.

Assay of HO-221 Concentration of HO-221 was measured by an HPLC system consisting of an LC3A constant flow pump and an SPD2A UV detector operating at 265 nm (Shimadzu Corp., Japan). Separations were performed on a reversed-phase Nova Pak C18 column (15 cm×3.9 mm i.d., 4 μm particle size, Waters Associates, Japan). The mobile phase consisted of acetonitrile–water (60:40, v/v). At a flow rate of 1.0 ml/min, the drug eluted at about 4 min at 30 °C.

Analysis of Plasma Data The maximum plasma concentration (C_{max}) and the time to reach this concentration (T_{max}) were obtained directly from the plasma concentration–time data. The area under the plasma concentration–time curve up to final sampling time (AUC) was determined by the trapezoidal rule. Statistical analyses were performd using Student's *t*-test.

Results and Discussion

Figure 2 shows the time course of particle-size reduction of HO-221 during the wet-bead milling under the conditions described in Experimental. The mean particle size of untreated HO-221 is 17.21±3.21 (mean±S.D.) μm, and this was reduced in accordance with length of milling time to 140 min. After milling, the final mean particle size was 0.453±0.023 μm, whereas by jet milling it was 4.15±0.46 μm. The final cumulative distribution curve of the wet-bead milling shows narrow distribution of particle size ranging from 0.1 to 1 μm. This final product, milled for 140 min, was used in the following experiments.

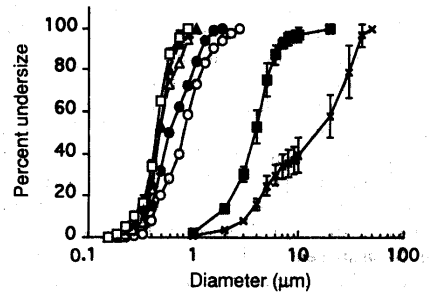


Fig. 2. Change of Particle Size Cumulative Distribution of HO-221 during Wet-Bead Milling

Suspension consisting of 30% (w/v) of HO-221 and 5% (w/v) of decaglycerin monolaurate was micronized with glass beads. Wet milling time: ○, 40 min; ●, 80 min; △, 100 min; ▲, 120 min; □, 140 min, ■, jet milled HO-221; ×, untreated HO-221. Each data point of wet milling for 140 min, jet milled and untreated HO-221 represents the mean±S.D. of three determinations, and others are the results of a single determination.

Figure 3 is an electron microscopic photograph of ground HO-221 particles after the wet-bead milling. They had round corner-edges and most were less than 0.5 μm, as shown by the particle size analyzer.

The achievable particle size seems to depend on the physicochemical properties of the compound to be milled. Low solubility and high melting point are thought to be preferable for micronization, empirically. The low solubility may be advantageous in suppressing crystal growth during and after the milling process, and the "hard" property (*i.e.*, a high melting point) may also be advantageous for crushing into small particles. If this speculation is correct, wet milling may be suitable not only for HO-221 but also for poorly soluble drugs which have a high melting point.

The value of the residue on ignition (JP, 1 g weight)[15] of the suspension was not more than 0.1%, which showed

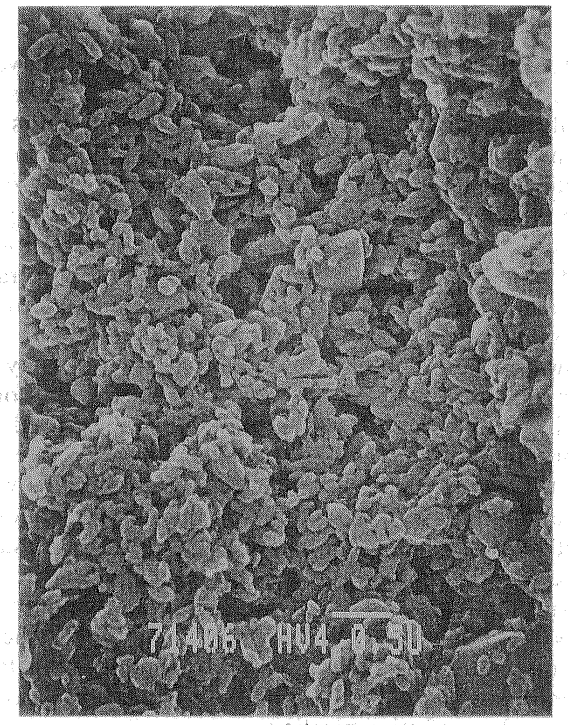


Fig. 3. Scanning Electron Microscope Photograph after Wet-Bead Milling

Bar: 0.5 μm.

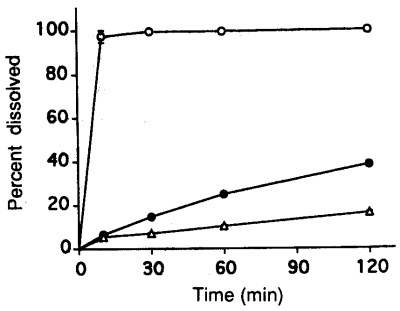


Fig. 4. Dissolution Profiles of Micronized HO-221 Preparations

○, wet milling suspension; ●, jet milled HO-221; △, untreated HO-221. Each point represents the mean±S.D. of three determinations.

TABLE I. Pharmacokinetic Parameters of HO-221 after Oral Administration of Micronized HO-221 Preparations in Rats at a Dose of 25 mg/kg

Preparation	n	C_{max} (μg/ml)	T_{max} (h)	AUC_{0-48h} (μg/ml·h)
Untreated HO-221	4	0.335±0.037	7.5±1.0	9.426±1.714
Jet milled HO-221	5	0.354±0.058	10.0±7.9	11.353±3.267
Wet milling suspension	5	0.564±0.129[b,c]	13.6±9.5	17.151±5.347[b]
Preparation B[a]	5	0.512±0.060[d]	14.0±9.2	16.410±1.890[d]

a) Lyophilized powder of wet milling suspension with the addition of sucrose palmitate at the HO-221/sucrose palmitate ratio of 1:2. *b*) Significantly different from untreated HO-221 ($p<0.01$). *c*) Significantly different from jet milled HO-221 ($p<0.05$). *d*) Significantly different from untreated HO-221 ($p<0.01$). Data are represented as mean±S.D.

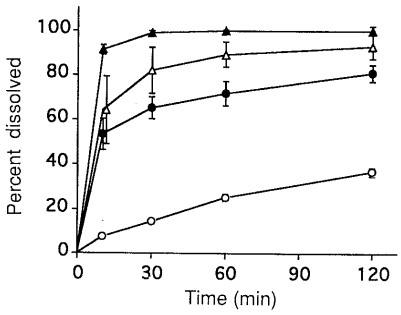


Fig. 5. The Effect of Sucrose Palmitate on Dissolution Profiles of Lyophilized HO-221

HO-221/sucrose palmitate ratio: ○, 1:0 (preparation A); ●, 1:0.5; △, 1:1, ▲, 1:2 (preparation B). Each point represents the mean±S.D. of three determinations.

the low contamination of the worn beads and/or container wall during the milling process.

The wet-bead milling increased the dissolution rate as shown in Fig. 4. The suspension after the wet-bead milling was rapidly dissolved within 10 min, whereas jet milled HO-221 dissolved slowly and slightly more rapidly than the untreated. As the solubility of HO-221 in 5% (w/v) of decaglycerin monolaurate aqueous solution is low (14.59 μg/ml at 37 °C) compared to its concentration in suspension (300 mg/ml), the effect of dissolved HO-221 in the suspension may be negligible. Several conditions were examined to measure the dissolution rate of the wet milling suspension, however, dissolution was too rapid to make this possible; the relationship between particle size and dissolution rate of HO-221 were therefore undetermined in this study. However, as defined by the Noyes–Whitney expression,[16] it is probable that increased surface area as a result of the particle-size reduction causes the enhancement of dissolution rate. The contribution of improved wettability by the milling with decaglycerin monolaurate is also believed to be an asset.

Table I shows the pharmacokinetic parameters of HO-221 after oral administration of the wet milling suspension, jet milled HO-221, untreated HO-221 and preparation B (*vide infra*) in rats. Reflecting the *in vitro* results, the wet milling suspension showed a higher C_{max} and greater AUC_{0-48h} than the other two. While the mean of each parameter for jet milled HO-221 was slightly higher than those for untreated HO-221, there were no significant differences between them.

A powder is the preferable form for further pharmaceutical processing (*i.e.*, granulation, tabletting or capsule filling) and for this reason a drying method of the wet milling suspension was sought. Figure 5 shows the dissolution profiles of HO-221 from the lyophilized powder of such a suspension. The powder without any treatment (preparation A) was slightly massive and sticky, and its dissolution rate was markedly decreased in comparison with that of the suspension before drying as shown in Figs. 4 and 5. Because of the poor water solubility of HO-221, the Van der Waals' interactions between the particles may essentially be increased with increase in the surface area of the particles, and this hydrophobic interaction may cause the particles to aggregate during the drying process. To prevent this aggregation, the addition of a detergent was evaluated.

Sucrose palmitate was chosen from a series of sucrose fatty acid esters which are powdery detergents because of its high hydrophilic properties; these are advantageous in preventing aggregation by hydrophobic interaction, thus allowing powder to dissolve quickly. As shown in Fig. 5, the dissolution rate was increased in accordance with the increased weight ratio of sucrose palmitate to HO-221. At the HO-221/sucrose palmitate ratio of 1:2 (preparation B), the dissolution profile was almost the same as that of the wet milling suspension. The pharmacokinetic parameters of HO-221 after oral administration of this preparation in rats are shown in Table I. There are no significant differences between before and after drying of the wet milling suspension.

To confirm the superiority of the wet-bead milling followed by the drying process presented here, the oral absorption of preparation B was compared in dogs with preparation C which has the same composition, but jet milled instead of wet-bead milled HO-221 was used. As shown in Fig. 6 and Table II, preparation B showed a

TABLE II. Pharmacokinetic Parameters after Oral Administration of Preparations in Dogs at a Dose of 25 mg/kg

Preparation	C_{max} (μg/ml)	T_{max} (h)	AUC_{0-240h} (μg/ml·h)
Preparation B[a]	0.479±0.131[c]	8.8± 1.8	49.809±10.186[c]
Preparation C[b]	0.287±0.097	24.0±26.8	23.800±14.513

a) Lyophilized powder of wet milling suspension with the addition of sucrose palmitate at the HO-221/sucrose palmitate ratio of 1:2. *b*) The same composition as preparation B excepting HO-221 was micronized by a jet mill. *c*) Significantly different from preparation C ($p<0.05$). Data are represented as mean±S.D. of five dogs.

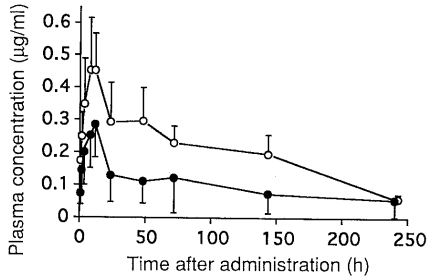


Fig. 6. Plasma Levels of HO-221 after Oral Administration of Preparation B (○) and Preparation C (●) in Dogs at a Dose of 25 mg/kg

Each point represents the mean±S.D. of five dogs.

significantly higher C_{max} ($p<0.05$) and greater $AUC_{0-240\,h}$ ($p<0.05$) than did preparation C: both were 2 times larger. As the same amount of detergents, decaglycerin monolaurate and sucrose palmitate, was contained in those preparations, the only difference between them was the milling process. Therefore, the increase of absorption in preparation B is due to the increased dissolution rate by the wet milling. It is almost certain that reduced particle size and improved wettability contribute to increase in this rate.

In conclusion, these findings suggest that wet-bead milling used for pharmaceutical preparations may enhance the oral absorption of extremely poor water soluble drugs. A study of the oral pharmacokinetics and bioavailability of HO-221 will be presented in a subsequent paper.

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