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In vitro biocompatibility studies with the experimental anticancer agent BIBX1382BS

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Abstract

The novel anticancer agent BIBX1382BS is a representative of the human epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors. BIBX1382BS, for parenteral use, is formulated pharmaceutically as a lyophilized product containing 100 mg BIBX1382BS per dosage unit. This in vitro study was performed to establish the optimal intravenous administration conditions (infusion concentration and infusion rate) for the forthcoming clinical absolute oral bioavailability study of BIBX1382BS. BIBX1382BS infusion solutions have a low pH in order to keep the substance in solution. We therefore decided to investigate the hemolytic and precipitation potential of the drug in vitro. Also, the ratio of formulation (*F*) solution volume and a blood simulans (*B*) volume necessary to reach the physiological pH, expressed as the *FB*-ratio, was determined in vitro. On the basis of the results obtained, it is advised to administer BIBX1382BS infusion at a concentration of 1 mg/ml and a maximum infusion rate of 10 ml/min. This article describes the in vitro biocompatibility screening program.

Keywords: BIBX1382BS; Biocompatibility; Hemolysis; Precipitation; *FB*-ratio

1. Introduction

BIBX1382BS (M_w 397.85, pyrimido[5,4-D]-pyrimidine-2,8-diamine,*N*8-(3-chloro-4-fluorophenyl)-*N*2-(1-methyl-4-piperidinyl)-2, Fig. 1) is a new anticancer agent which exerts its activity by a potent and selective inhibition of the human epidermal growth factor receptor (EGFR) tyrosine kinase. As the anti-tumour activity of BIBX1382BS most likely depends on a continuous inhibition of the EGFR function, oral administration is aimed for as the primary route of administration. To determine the absolute oral bioavailibility of BIBX1382BS, the plasma con-

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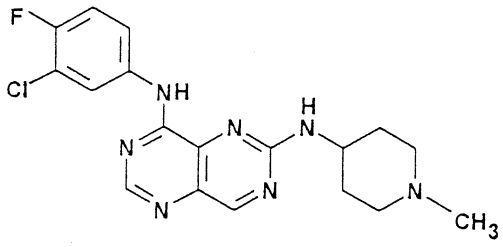

Fig. 1. Structural formula of BIBX1382BS.

centration time curves (AUCs) after oral and intravenous administration will be compared in a bioavailability study. For the conduct of this study, an intravenous dosage form of BIBX1382BS was designed. The drug for intravenous use is formulated as a lyophilized product containing 100 mg of BIBX1382BS as its HCl salt and 250 mg of D-mannitol as bulking agent. The freeze-dried product is to be reconstituted with 10 ml of water for injection (WfI), resulting in a solution containing 10 mg/ml BIBX1382BS. The reconstituted product has a pH of ~ 2, which is necessary to keep BIBX1382BS solubilized. Below pH 3.0 BIBX1382BS (pK_{a_1} 3.0, pK_{a_2} 9.2) is present in its dicationic form. Intravenous administration of solutions with a low pH can give cause to various adverse side-effects. Besides vascular irritation and phlebitis at the injection site, and precipitation of the solubilized drug can occur as a consequence of the sudden pH-change of the infusion solution entering the bloodstream, giving rise to erratic or reduced bioavailibility (Yalkowsky et al., 1983; Li et al., 1998). Furthermore, hemolysis or the destruction of red blood cells in the bloodstream can occur resulting in the release of hemoglobin into the plasma which is associated with vascular irritation and thrombophlebitis, and even anemia, jaundice, kernicterus and acute renal failure (Krzyzaniak et al., 1997a,b).

In this study, the optimal administration conditions in terms of BIBX1382BS infusion solution concentration and infusion rate for the forthcoming bioavailability study were determined using an in vitro biocompatibility screening program composed of the determination of the hemolytic and precipitation potential. Also, the ratio of formulation (F) solution volume and a blood simulans (B) volume necessary to reach the physiological pH, expressed as the FB-ratio, was determined.

2. Experimental

2.1. Chemicals and materials

BIBX1382BS was synthesized by Boehringer Ingelheim Pharma KG and provided through the New Drug Development Office-Oncology (NDDO-Oncology, Amsterdam, The Netherlands). All chemicals used were of analytical grade and were used without further purification. Diazepam CF (5 mg/ml diazepam in propylene glycol/ethanol/water 40/10/50% v/v/v) was obtained from Centrafarm, Etten-Leur, The Netherlands. Cisplatine TEVA was obtained from TEVA Pharma, Amsterdam, The Netherlands. BIBX1382BS 100 mg/vial lyophilized product, water for injection (WfI), 0.9% (w/v) sodium chloride for infusion (normal saline) and isotonic Sorensen's buffer (1/15 M phosphate buffer at pH 6.8) were manufactured in-house. Before use, isotonic Sorensen's buffer was adjusted to pH 7.4 with 1 M sodium hydroxide. Fresh heparinized blood with a labeled hematocrit of 50% for the whole blood hemolysis study was purchased from the local blood bank (CLB, Amsterdam, The Netherlands). All experiments were conducted at room temperature (20–25°C).

2.2. Preparation of infusion solutions

BIBX1382BS 100 mg/vial lyophilized product was reconstituted with 10 ml of WfI resulting in a 10 mg/ml solution with a pH of ~ 2. Infusion solutions in concentrations of 1 mg/ml (pH 2.7), 0.5 mg/ml (pH 3.0) and 0.1 mg/ml (pH 3.3) BIBX1382BS were prepared by diluting the reconstituted product with normal saline.

2.3. Precipitation studies

The potential of precipitation of BIBX1382BS from infusion solutions was examined using the dynamic flow model described by Yalkowsky et al. (1983). The testing apparatus consisted of a

Model 711 syringe pump (IVAC, San Diego, USA), a Model 501Dz peristaltic pump (Watson Marlow, Rotterdam, The Netherlands) and a Model UV/VIS 918 spectrophotometer (GBC Scientific Equipment, Victoria, Australia) equipped with a flow cell cuvet with a fill capacity of ~3 ml and a LEO personal computer and Epson LX-400 plotter. Silicone tubing with a length of 75 cm and an internal diameter of 1.6 mm was applied (Watson Marlow, Rotterdam, The Netherlands). Isotonic Sorensen's buffer adjusted to a pH of 7.4 was used as a blood simulator and was pumped through the system at a flow rate of 6 ml/min. Light scattering (opacity) due to precipitation was measured at a wavelength of 500 nm. BIBX1382BS infusion solutions at concentrations of 10, 1 and 0.1 mg/ml were infused at rates of 0.15, 0.3, 0.6 and 1.5 ml/min (corresponding to formulation:buffer ratios of 0.025, 0.05, 0.1 and 0.25, respectively) in the running buffer flow at a distance of 25 cm from the flow cell cuvet. Also, a blank infusion solution containing only blank vehicle in the same concentrations as in the BIBX1382BS 10 mg/ml infusion solution was tested. As a positive control, precipitation induced by diazepam at a concentration of 5 mg/ml formulated in a mixture of 40/10/50% (v/v/v) propylene glycol/ethanol/water (PEW) was determined using the same procedure as for the BIBX1382BS infusion solutions, including an additional formulation:buffer ratio of 0.014. Precipitation was measured in quadruplicate as the plateau opacity induced by infusion of a formulation solution.

2.4. Determination of FB-ratios

The volumes of a blood simulans to be added to BIBX1382BS infusion solutions in concentrations of 1, 0.5 and 0.1 mg/ml necessary to reach a physiological pH of 7.4 were determined by titration of 50.0 ml of infusion solution with isotonic Sorensen's buffer (adjusted to pH 7.4), using a Model 682 Titroprocessor and a Model 665 Dosimat (Metrohm, Herisau, Switzerland). In this way, an indication is obtained about the in vivo buffer capacities of the BIBX1382BS infusion solutions upon intravenous administration. As a reference, a commercially available cisplatin infusion solution with a known low pH (cisplatine TEVA, pH 4.4) at a concentration of 0.1 mg/ml was prepared according to the manufacturer's preparation instruction and processed like the BIBX1382BS infusion solutions. The ratio of the volume of formulation solution titrated and the volume of isotonic Sorensen's buffer necessary to reach the physiological pH, expressed as the *FB*-ratio, was calculated using Eq. (1):

$$\textit{FB}\text{-ratio} = (\text{volume infusion solution}) \times /(\text{volume Sorensen's buffer}) \quad (1)$$

2.5. Hemolysis studies

The potential of hemolysis upon BIBX1382BS infusion solution administration was evaluated using both the static and dynamic in vitro test models as described by Ward and Yalkowsky (1993), Krzyzaniak et al. (1997a), Krzyzaniak et al. (1997b) and Krzyzaniak and Yalkowsky (1998). For both models, BIBX1382BS infusion solution at a concentration of 1 mg/ml in normal saline was used. Also, a blank infusion solution containing only the BIBX1382BS vehicle was tested. All experiments were run in duplicate. Fresh, heparinized human blood with a labeled hematocrit content of 50% was used throughout the experiments. For the static model, 25, 50 and 125 μl of BIBX1382BS infusion solution were added to 500 μl of blood, resulting in formulation:blood ratios of 0.05, 0.1 and 0.25, respectively. A blood-formulation contact time of 5 s by manual shaking was employed. For the dynamic model, BIBX1382BS infusion solution was infused at a rate of 0.3, 0.6 and 1.5 ml/min using a Model 711 syringe pump (IVAC, San Diego, USA) in a running blood flow set at 6 ml/min using a Model 501Dz peristaltic pump (Watson Marlow, Rotterdam, The Netherlands) resulting in formulation:blood ratios of 0.05, 0.1 and 0.25, respectively. The contact time of blood and formulation was set at 5 s applying a silicone tube with a length of 25 cm and an internal diameter of 1.6 mm (Watson Marlow, Rotterdam, The Netherlands). For both the static and dynamic

model, the hemolytic reaction was quenched by the addition of 50 ml of normal saline. Subsequently, an aliquot of 3 ml of each solution was centrifuged at 3000 rpm for 10 min and the absorption (A) of the supernatant at 540 nm was determined using a Model UV/VIS 918 spectrophotometer (GBC Scientific Equipment, Victoria, Australia) equipped with a LEO personal computer and an Epson LX-400 plotter. As a positive control, hemolysis induced by 40/10/50% (v/v/v) PEW was determined using the same procedure as for the infusion solution. The baseline hemolysis level was determined by treating normal saline as the infusion solution at all formulation:blood ratios. The 100% hemolysis level was determined by diluting the blood volume applied in the static and dynamic model with distilled water instead of normal saline. The percentage hemolysis induced by the infusion solution and PEW vehicle was calculated using Eq. (2) (Krzyzaniak and Yalkowsky, 1998):

$$\%\text{Hemolysis} = (A_{\text{infusion}} - A_{\text{saline}})/(A_{100\%} - A_{\text{saline}}) \times *100\% \quad (2)$$

3. Results and discussion

3.1. Precipitation studies

Infusion of a pH-based solubilized drug formulation into the bloodstream results in a sudden and fast dilution of the drug solution. The accompanying shift in pH due to the buffering capacity of blood can result in precipitation of the solubilized component. Intravascular precipitation is related to pain and phlebitis at the injection site as well as altered bioavailability. It has been demonstrated that the dynamic in vitro precipitation testing method shows a good relationship with the in vivo situation and is therefore useful in the determination of 'safe' infusion solution concentrations and infusion rates (Davio et al., 1991). BIBX1382BS infusion solutions at the highest concentration of 10 mg/ml and additionally at 1 and 0.1 mg/ml were subjected to in vitro precipitation testing. Given a venous blood flow of 40 ml/min and hypothetical infusion rates of 1, 2, 4 and 10 ml/min, formulation:buffer ratios of 0.025, 0.05, 0.1 and 0.25 were examined. It is assumed that the formulation:blood composition in the in vivo situation is determined by the infusion rate and can be estimated with knowledge of the venous blood flow at the injection site (Ward et al., 1993; Krzyzaniak et al., 1997a). As reference, a diazepam formulation with known precipitation when injected into the bloodstream at rates exceeding 1 ml/min, corresponding to a formulation:buffer ratio of 0.025, was selected. Any opacity detected with diazepam at this formulation:buffer ratio in the in vitro simulation experiment was taken as a threshold value above which adverse effects (e.g. pain, phlebitis) are likely to occur (product information Diazepam CF Injectable; Li et al., 1998). From Table 1 it can be seen that diazepam precipitation occurred at all formulation:buffer ratios examined with a threshold value of ~ 0.2 at a formulation:buffer

Table 1
Results of in vitro precipitation testing

Infusion solution	Mean opacity as a result of precipitation at formulation:buffer ratio				
	0.014	0.025	0.05	0.10	0.25
BIBX1382BS 10 mg/ml	n/e[a]	0.03 ± 0.01	0.02 ± 0.01	0.08 ± 0.1[b]	0.2 ± 0.2[b]
BIBX1382BS 1 mg/ml	n/e	<0.01	<0.01	<0.01	<0.01
BIBX1382BS 0.1 mg/ml	n/e	<0.01	<0.01	<0.01	<0.01
Blank BIBX1382BS	n/e	<0.01	<0.01	<0.01	<0.01
Diazepam 5 mg/ml	0.08 ± 0.1	0.2 ± 0.01	0.5 ± 0.1	1.7 ± 0.06	2.4 ± 0.05

[a] n/e, not executed.
[b] Visual precipitation.

Table 2
FB-ratios of BIBX1382BS and cisplatin infusion solutions

Infusion solution (mg/ml)	*FB*-ratio
BIBX1382BS 1	5.1 ± 0.1
BIBX1382BS 0.5	12.5 ± 0.4
BIBX1382BS 0.1	33.5 ± 3.1
Cisplatin 0.1	62.5

ratio of 0.025. A similar precipitation profile of diazepam was reported by Li et al. (1998). The 10 mg/ml BIBX1382BS solution showed visual precipitation in the tubing at formulation:buffer ratios of 0.10 and 0.25, confirmed by the degree of opacity measured at these formulation:buffer ratios. Also, BIBX1382BS infusion solutions at 10 mg/ml showed some opacity at the lower infusion rates corresponding to formulation:buffer ratios of 0.025 and 0.05. At all other BIBX1382BS concentrations and formulation:buffer ratios the opacity induced by the drug infusion solutions did not differ significantly from those obtained with blank infusion solution or normal saline and were well below the threshold value of 0.2 as determined with the diazepam formulation (Table 1). On the basis of these results, adverse side-effects as a consequence of precipitation of drug substance from the BIBX1382BS solutions are likely to occur when administering BIBX1382BS infusion solutions at a concentration of 10 mg/ml with an infusion rate ≥ 4 ml/min.

3.2. FB-ratios

In Table 2 the results of the in vitro determination of the *FB*-ratios of BIBX1382BS infusion solutions are given. A cisplatin infusion solution was choosen as a reference because of its low pH value of 4.4 at the tested concentration of 0.1 mg/ml. BIBX1382BS infusion solution at a concentration of 10 mg/ml was not examined because of the precipitation upon infusion as determined in the in vitro precipitation experiment described above. As can be seen, the *FB*-ratio for the cisplatin infusion solution is significantly larger compared to the *FB*-ratios obtained with the BIBX1382BS infusion solutions, thus indicating higher in vivo buffer capacities of the BIBX1382BS solutions. However, when extrapolating the *FB*-ratios obtained to the clinical situation assuming a venous blood flow of 40 ml/min, BIBX1382BS can theoretically be administered at infusion rates up to 204 ml/min (1 mg/ml BIBX1382BS), 500 ml/min (0.5 mg/ml BIBX1382BS) and 1340 ml/min (0.1 mg/ml BIBX1382BS) without change of the pH of the blood at the injection site. In conclusion, no adverse effects are expected as a consequence of the low pH of BIBX1382BS infusion solutions at concentrations up to 1 mg/ml.

3.3. Hemolysis studies

Studies conducted so far to determine the hemolytic potential of pharmaceutical formulations are almost exclusively focused on the intravenous bolus injection and not on the continuous infusion of pharmaceutical solutions (Krzyzaniak et al., 1997a; Krzyzaniak and Yalkowsky, 1998; Ward and Yalkowsky, 1993; Gupta et al., 1994). Although infusion solutions generally contain highly diluted drug vehicles and relatively low drug concentrations and are therefore less expected to induce blood cell destruction, the hemolytic potential of such a solution infused over a longer period of time should be evaluated as a part of biocompatibility studies of a new formulation, certainly if the pH of the infusion solution differs significantly from the physiological pH. We studied the hemolytic potential of a BIBX1382BS infusion solution at a concentration of 1 mg/ml, selected from the results obtained with the in vitro precipitation testing and *FB* ratio determination. Instead of a blood–formulation contact time of 1 s, proposed by Krzyzaniak et al. (1997a,b) as physiologically realistic after an intravenous injection, a contact time of 5 s was applied to correct for the continuous exposure during an intravenous infusion, assuming that after this period the formulation will be diluted significantly in the central compartment. Based on a venous blood flow of 40 ml/min and hypothetical infusion rates of 2, 4 and 10 ml/min, formulation:buffer ratios of 0.05, 0.1 and 0.25 were examined. From Table 3 it can be seen that for

Table 3
Results of in vitro hemolysis testing

Infusion solution	Mean hemolysis (%) at formulation:buffer ratio			
	Model	0.05	0.10	0.25
BIBX1382BS 1 mg/ml	Static	0	0.1 ± 0.2	0.6 ± 0.4
	Dynamic	0	0.6 ± 0.2	0
Blank BIBX1382BS	Static	0.3 ± 0.3	0.5 ± 0.4	0.3 ± 0.2
	Dynamic	0.4 ± 0.5	0.3 ± 0.5	0
40/10/50% v/v/v propylene glycol/ethanol/water (PEW)	Static	5.7 ± 1.3	28.4 ± 0.09	53.7 ± 1.5
	Dynamic	13.3 ± 0.3	22.8 ± 0.6	39.8 ± 1.7

both the static and the dynamic model the degrees of hemolysis seen with BIBX1382BS infusions did not differ significantly with those obtained with blank infusion solutions (all $< 1\%$ hemolysis). Hemolysis levels seen with 40/10/50% (v/v/v) PEW are comparable with percentages found earlier. Also, the higher percentages of hemolysis obtained with the static in vitro model compared with the dynamic model are in agreement with data in the literature (Krzyzaniak et al., 1997a). Ward and Yalkowsky (1993) suggested that the static model may not be appropriate as a predictive model for the hemolytic potential because of the occurrence of false-positive results. For the in vitro dynamic hemolysis model it was reported that this method is a good predictor of phlebitis as a result of hemolysis upon injection of pharmaceutical vehicles (Krzyzaniak et al., 1997a). In this study no hemolysis was detected in neither the static nor the dynamic model with BIBX1382BS and blank infusion solutions. Therefore, it is not expected that BIBX1382BS infusion solutions will cause red blood cell destruction upon administration, at an infusion concentration of 1 mg/ml and infusion rates up to 10 ml/min.

4. Conclusions

On the basis of the results of the in vitro precipitation, *FB*-ratio determination and hemolysis experiments performed BIBX1382BS should be administered to the patient in infusion concentrations equal or below 1 mg/ml at infusion rates up to 10 ml/min. Although the data were obtained with in vitro testing which cannot mimic the in vivo situation completely (e.g. protein binding, venous blood flow), the biocompatibility screening program used gives a valid indication of the way of administrating BIBX1382BS, a pH solubilized drug, in clinical practice. Thus far, no adverse effects as a consequence of the intravenous administration of BIBX1382BS in the ongoing clinical study have occurred.

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