

# Pharmacokinetic characterization of CK2 inhibitor CX-4945

You Hwa Son · Jin Sook Song · Seong Hwan Kim ·  
Jiyeon Kim

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**Abstract** Over-expression of protein kinase CK2 is highly linked to the survival of cancer cells and the poor prognosis of patients with cancers. CX-4945, a potent and selective orally bioavailable ATP-competitive inhibitor of CK2, inhibits the oncogenic cellular events such as proliferation and angiogenesis, and the increase of tumor growth in mouse xenograft model. In this study, the pharmacokinetic information about CX-4945 was provided; at 10  $\mu$ M, CX-4945 with high stability in human and rat liver microsome exhibited low percentage of inhibition (<10 %) in CYP450 isoforms (1A2, 2C19, 3A4), but considerable inhibition ( $\sim$ 70 %) in CYP450 2C9 and 2D6. In hERG potassium channel inhibition assay, CX-4945 exhibited relatively low inhibition rate. Additionally, CX-4945 showed high MDCK cell permeability ( $>10 \times 10^{-6}$  cm/s) and above 98 % of plasma protein binding in the rat. After intravenous administration,  $V_{ss}$  (1.39 l/kg) and extremely low CL (0.08 l/kg/h) were observed. Moreover, orally administrated CX-4945 showed high bioavailability ( $>70$  %) and these data might be related to the MDCK cell permeability results.

**Keywords** CK2 · CX-4945 · Pharmacokinetics · Druggability

## Introduction

As a serine/threonine kinase, CK2 is ubiquitously and constitutively expressed, and exists as a tetramer which is composed of 2 catalytic ( $\alpha$  and  $\alpha'$ ) and 2 regulatory ( $\beta$  and  $\beta'$ ) subunits. CK2 has been known to play an important role in the regulation of cell cycle, differentiation, and proliferation (Litchfield 2003; Seldin and Leder 1995; Landesman-Bollag et al. 2001) that are controlled by the sophisticated cross-talk between multiple signaling pathways including PI3K/Akt, Wnt, and NF- $\kappa$ B (Buerra and Issinger 2008; Duncan and Litchfield 2008). CK2 induction observed in many cancers is highly related to poor prognosis in the cancer progression (Landesman-Bollag et al. 2001; O-charoenrat et al. 2004; Laramas et al. 2007; Pistorius et al. 1991; Stalter et al. 1994). CK2 also regulates angiogenesis. Under hypoxia condition, CK2 was induced and its induction subsequently stimulated hypoxia-inducible transcription factor 1 alpha (HIF-1 $\alpha$ ) activity (Mottet et al. 2005) and proliferation of epithelial cells (Ljubimov et al. 2004). In this regard, as the over-expression of CK2 in cancer cells can be represented as a hallmark of maintaining oncogenic signaling, therapeutic approaches for CK2 inhibition exploited.

Apparently, antitumor activity of CK2 inhibition has been reported in several studies (Wang et al. 2005; Slaton et al. 2004) and several ATP-competitive inhibitors of CK2 have been identified (Sarno and Pinna 2008; Lopez-Ramos et al. 2010; Sandholt et al. 2009; Hung et al. 2009). Among them, polyhalogenated heteroaryl 4,5,6,7-tetrabromo-1H-benzotriazole and 2-dimethylamino-4,5,6,7-tetrabromo-

Y. H. Son · S. H. Kim (✉) · J. Kim (✉)  
Laboratory of Translational Therapeutics, Korea Research  
Institute of Chemical Technology, P.O. Box 107, Yuseong-gu,  
Daejeon 305-600, Republic of Korea  
e-mail: hwan@kriict.re.kr

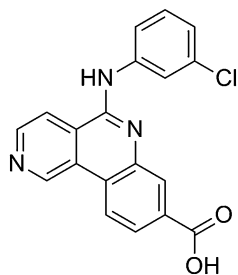
J. Kim  
e-mail: yeon@kriict.re.kr

J. S. Song  
Drug Discovery Platform Technology Team, Bio-Organic  
Science Division, Korea Research Institute of Chemical  
Technology, P.O. Box 107, Yuseong-gu, Daejeon 305-600,  
Republic of Korea

1H-benzimidazole, have been the commercially used for CK2-targeted experiments, but these compounds have not been developed further because of low bioavailability (Battistutta et al. 2001; Pagono et al. 2008). However, Cyline Pharmaceuticals has recently developed a first-in-class selective CK2 $\alpha$  inhibitor coded CX-4945 (5-(3-chlorophenylamino) benzo[*c*][2,6] naphthyridine-8-carboxylic acid; Fig. 1) with oral bioavailability and it has entered the clinical trials (clinicaltrials.gov; identifier: NCT00891280 & NCT01199718) (Pierre et al. 2011; Kim and Kim 2012).

In preliminary reports, CX-4945 already exhibited anti-proliferative and anti-angiogenic activity in human cancer cells. In a broad spectrum of human breast cancer cell lines, CX-4945 exerted nanomolar scales (1 nM) of IC<sub>50</sub> against CK2 $\alpha$  and CK2 $\alpha'$  and suppressed PI3K/Akt signaling in cancer cells (Siddiqui-Jain et al. 2010). Moreover, in this report, CX-4945 showed the regulatory effect in cell cycle progression through the dephosphorylation of p21 and p27 cell cycle inhibitor proteins and the apoptotic activity through caspase 3/7 inductions. In vivo administration of CX-4945 exerted partial or complete antitumor efficacy in BT-474 breast cancer and BxPC-3 pancreatic cancer-inoculated xenograft models. CX-4945 also exhibited an anti-inflammatory effect in human inflammatory breast cancer (Drygin et al. 2011). Among the human breast cancer, inflammatory breast cancer (IBC) is a characterized by over-expression of pro-inflammatory cytokines, angiogenic chemokines, and growth factors such as interleukin-6 (IL-6), IL-8, VEGF (van Golen et al. 2000). These pro-inflammatory factors might be modulated by a potential upstream regulator CK2 (Parhar et al. 2007; Ruzzene and Pinna 2010). Treatment of CX-4945 to the SUM-149PT IBC cell line reduced the secretion of IL-6 and oral administration also resulted in the reduction of IL-6 in human plasma.

Safety and efficacy are major considerations in the drug development stage. Because good in vitro activity may not reflect good in vivo efficacy, in-depth studies of pharmacokinetics and drug metabolism should be carefully performed. Despite of well-organized pharmacological data of CX-4945, its pharmacokinetic profile has been simply described (Pierre et al. 2011). Thus, in this



**Fig. 1** Chemical structure of CX-4945

study, we discussed the results reported by Pierre et al. (2011) and provided the additional pharmacokinetic profiles of CX-4945 that would be helpful to understand its druggability (or drug feasibility) for further development of CK2 inhibitors.

## Materials and methods

### Chemicals and animals

CX-4945 was purchased from Sequoia Research Products (Pangbourne, UK). Sprague-Dawley (SD) rats (male,  $n = 3$ ) were purchased from Orient Bio Inc. (Seongnam, Korea) and housed in an air-conditioned room at a temperature of  $23 \pm 2$  °C. Before the experiments, rats were fasted for 12 h except for water supply. All experimental procedures for animal care and housing were in accordance with the KRICT Animal Care and Use Committee.

### NADPH-dependent metabolic stability of CX-4945

Liver metabolic stability of CX-4945 was determined in human and rat liver microsomes as described in a previous study (Song et al. 2011a, b). Briefly, CX4945 (10  $\mu$ M) was mixed with human or rat liver microsomes (0.5 mg/ml; BD Biosciences Gentest, CA) in 100 mM potassium phosphate buffer (pH 7.4) and incubated at 37 °C for 5 min. The reaction was initiated by NADPH regeneration solution (BD Biosciences) and terminated by three times volume of ice-cold acetonitrile with imipramine (80 ng/ml) as internal standard at single-time-point 30 min. After pre-treatment of biological samples with vortex and centrifuge, the samples were analyzed by LC/MS/MS system.

### CYP450 inhibition assay

As described in a previous study (Song et al. 2011a, b), the potential of CX-4945 to inhibit major human cytochrome P450 (CYP450) enzymes was evaluated using Vivid CYP450 screening kit (Invitrogen, CA); the % inhibition of CX-4945 for CYP1A2, 2C9, 2C19, 2D6, and 3A4 isoforms was measured by fluorescence detection.

### Automated patch clamp

HEK293 cells stably expressing hERG (HEK293-hERG cells; Genionics, Switzerland), were used to assess cardiac toxicity. The cells were grown at 37 °C and 5 % CO<sub>2</sub> in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal calf serum (Gibco, Invitrogen, UK) and 1 M glutamine. When cells were more than 80 %

confluent, the medium was removed using an aspirator and cells were washed twice with PBS. After detaching cells from culture flasks, counted cells ( $2 \times 10^5$  cells/ml) with medium. Then, cells that were centrifuged, resuspended in 150  $\mu\text{L}$  of extracellular solution and applied to the AutoPatch system (PatchXpress 7000A, Molecular Devices, Sunnyvale, CA). Extracellular solution was prepared as the following; HBSS (Invitrogen; Carlsbad, CA) was the standard external bath solution used to record hERG currents and had the following ionic composition (in mM): NaCl, 138; KCl, 5.3;  $\text{CaCl}_2$ , 1.3;  $\text{MgCl}_2$ , 0.5; Glucose, 5.6; HEPES, 5;  $\text{KH}_2\text{PO}_4$ , 0.44;  $\text{MgSO}_4$ , 0.41;  $\text{NaHCO}_3$ , 4;  $\text{Na}_2\text{HPO}_4$ , 0.3; pH 7.4 with NaOH/HCl. The standard internal solution had the following ionic composition (in mM): KCl, 130; MgATP, 5;  $\text{MgCl}_2$ , 1.0; HEPES, 10; EGTA 5, pH 7.2 with KOH. The experiment was initiated with a buffer control and then CX-4945 was applied. The tail current was monitored continuously and the data were saved automatically into the PatchXpress database. The  $\text{IC}_{50}$  values were derived automatically from curve-fitting plots of CX-4945.

#### Cell permeability assay

Cell permeability assay was performed as described in a previous study (Park et al. 2011). Madin-Darby Canine Kidney (MDCK) cells were grown in DMEM supplemented with 10 % fetal bovine serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin in humidified 37 °C incubator with 5 %  $\text{CO}_2$  atmosphere. MDCK cells were seeded at a density of  $6 \times 10^4$  cells/ $\text{cm}^2$  in a 12-well transwell plate. The medium was changed the day after seeding and every other day thereafter by adding to both apical and basolateral compartments. Cell monolayers were used after 3–4 day incubation to ensure cell monolayer integrity. The transepithelial electrical resistance (TEER) values were measured using an EVOM epithelial tissue voltammeter (World Precision Instruments, FL). MDCK cell monolayers with the TEER values above 700 ohms were preincubated in transport buffer (HBSS with 10 mM glucose and 25 mM HEPES adjusted to pH 7.4) for 30 min at 37 °C. CX-4945 in transport buffer (50  $\mu\text{M}$ ) were added to the apical side (for apical to basolateral permeability measurements). For 120 min with intervals of each 30 min, 200  $\mu\text{l}$  of transport buffer were taken and added with fresh buffer from basolateral side. The aliquots were stored at  $-20$  °C until LC/MS/MS analysis. At the completion of all experiments, TEER was measured to ensure cell monolayer integrity. No effect of CX-4945 in transport buffer (50  $\mu\text{M}$ ) on cell viability has been observed. The apparent permeability coefficients ( $P_{\text{app}}$ , cm/s) were calculated using following equation:  $P_{\text{app}} = (dQ/dt)/(A \times C_0)$ , where  $dQ/dt$  is the rate of permeation across the monolayer, A is the

surface area of the monolayer ( $0.33 \text{ cm}^2$ ), and  $C_0$  is the initial concentration in the donor compartment. Metoprolol (50  $\mu\text{M}$ ) and atenolol (50  $\mu\text{M}$ ) were used as markers to show high and low permeability, respectively.

#### Plasma protein binding assay

Plasma protein binding rate of CX-4945 in rat was evaluated as described in a previous study (Park et al. 2011). Briefly, spiked CX-4945 (2  $\mu\text{g/ml}$  as a final concentration) in 500  $\mu\text{l}$  of rat plasma was incubated for 30 min at 37 °C prior to ultrafiltration. A 50  $\mu\text{l}$  aliquot of incubated solution was collected for total concentration calculation and a 350  $\mu\text{l}$  aliquot was placed in Amicon Ultra-0.5 centrifugal filter devices with 30,000 nominal molecular weight limit (Millipore, MA) and centrifuged at  $3,000 \times g$  for 30 min at 37 °C to separate the protein bound CX-4945 from free form.

#### In vivo pharmacokinetics

In vivo pharmacokinetics was performed as described in a previous study (Park et al. 2011). The rats were cannulated with polyethylene tubing (PE-50, Intramedic, BD Bioscience, MD) in the femoral vein under ketamin-administered anesthesia. After 1 day recovery from anesthesia and surgery, CX-4945 was dissolved in a mixture of DMSO/PEG400/distilled water (0.5:4:5.5) and administered to rats by a bolus injection via the femoral vein at dose of 10 mg/kg and oral gavage at dose of 10 mg/kg. Blood samples were collected via the femoral vein pre-dose and 2, 10, 30 min and 1, 2, 4, 6, 8, 24 h for the case of intravenous administration, or 15, 30 min and 1, 2, 4, 6, 8, 24 h for the oral administration case after CX-4945 administration. After centrifugation of blood samples, 100  $\mu\text{l}$  aliquots of plasma samples were collected and stored at  $-70$  °C before LC/MS/MS analysis. The concentrations of CX-4945 were determined by an LC/MS/MS method. In brief, 50  $\mu\text{l}$  aliquots of the biological samples and 450  $\mu\text{l}$  acetonitrile containing internal standard (imipramine 80 ng/ml) were mixed. After vortexing and centrifuging at 10,000 rpm and 4 °C, the 5  $\mu\text{l}$  of supernatants were directly injected to LC/MS/MS system. The chromatographic system consisted of an Agilent 1100 series HPLC and Hydrosphere C18 column (3  $\mu\text{m}$ ,  $2.0 \times 50$  mm; YMC) using a mobile phase with 90 % acetonitrile with 10 mM ammonium formate buffer at a flow rate of 0.3 ml/min. The eluent was introduced directly into the tandem quadrupole mass spectrometer (API 4000 Q TRAP, AB SCIEX, CA). Multiple reaction monitoring (MRM) mode based on most abundant product ions were at  $m/z$  418.28  $\rightarrow$  72.00 for CX-4945, and  $m/z$  281.3  $\rightarrow$  86.1 for internal standard with the mass spectrometry condition through the turbo gas, 50 psi; ion

**Table 1** Percentage of CYP inhibition of CX-4945 at 10  $\mu\text{M}$ 

CYP isoforms	1A2	2C9	2C19	2D6	3A4
CX-4945	5.32	69.78	5.86	69.78	7.10
Inhibitor	95.72 ( $\alpha$ -naphthoflavone)	81.80 (sulfaphenazole)	72.70 (ketoconazole)	81.80 (quinidine)	96.13 (ketoconazole)

spray voltage, 5500 V; temperature, 400 °C. The standard curve was linear in the concentration range of 7.8–8,000 ng/ml. The pharmacokinetic parameters were analyzed by a non-compartmental method using WinNolin software (Pharsight, CA). The area under the plasma concentration–time curve (AUC) and the area under the first moment curve were calculated using the trapezoidal rule extrapolated to infinity. The terminal elimination half-life ( $t_{1/2}$ ), the systemic clearance, mean residence time (MRT) and volume of distribution at steady state ( $V_{ss}$ ) were obtained. The extent of absolute oral bioavailability (F) was estimated by comparing the AUC values after intravenous and oral administration of the same dose of CX-4945. The peak plasma concentration ( $C_{max}$ ) and the time to reach  $C_{max}$  ( $T_{max}$ ) after oral administration were obtained by visual inspection from each rat's plasma concentration–time plot for CX-4945. All data were expressed as the mean  $\pm$  standard deviation (SD).

## Results and discussion

### Liver microsomal metabolic stability

In vitro metabolic stability is the key factor that influences on the pharmacokinetic parameters such as the clearance, half-life, and oral bioavailability of drugs. Metabolic stability assay revealed that the human and rat liver microsomal stability of CX-4945 were  $78.0 \pm 14.8$  and  $82.6 \pm 0.1$ , respectively.

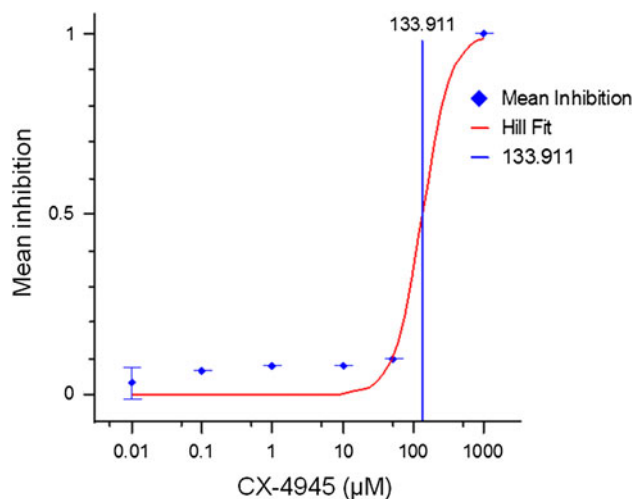
### CYP450 inhibition

The phase I drug metabolism process involves the reaction of oxidation, reduction, and hydrolysis and is induced by catalysis of a number of metabolic enzymes. Among these enzymes, the most important enzyme family, the cytochrome P450 (CYP) super-family has more than 500 different isoforms of CYP in humans, plants and animals (Rendic and Di Carlo 1992), but only few isoforms of CYP (1A2, 2A6, 2C8, 2C9, 2C19, 2D6, 3A4, and etc.) are associated with drug–drug interaction (DDI) studies (Rodrigues 1999). In particular, CYP3A4 is the most abundant and significant CYP isoform in human liver and it plays a pivotal role in DDI and drug-clearance during drug development

processes (Rodrigues 1999; Thummel and Wilkinson 1998). In an early published group, they have just described that CX-4945 showed minimal inhibition of five CYP450 isoforms (CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4) (Pierre et al. 2011). As shown in Table 1, in this study, CX-4945 exerted relatively weak (CYP1A2; 5.32 %, CYP2C19; 5.86 %, CYP3A4; 7.10 %) enzyme inhibition activities at 10  $\mu\text{M}$ . However, % CYP inhibition activity of CYP2C9 and CYP2D6 was above 60 %.

### hERG potassium channel inhibition

Recently, several preclinical drug candidates in drug development processes or on sale drugs have been resulted in non-approval or withdrawals from the market because of the drug-induced cardiac arrhythmia (Belardinelli et al. 2003; Redfern et al. 2003). Therefore, the inhibition level of the hERG potassium channel related to cardiac action potential is currently the most surrogate parameter to predict cardiac safety of novel chemical entities. In a previous study, CX-4945 did not show any significant inhibition of the hERG channel (<10 % inhibition at 0.1, 1.0, and 10.0  $\mu\text{M}$ ) in the patch clamp assay. In this study, to confirm the previous data, we tested the effect of CX-4945 on the hERG potassium channel by automated patch clamp. As shown in Fig. 2, CX-4945 showed extremely low inhibition effect and more than 100  $\mu\text{M}$   $\text{IC}_{50}$  (133.91  $\mu\text{M}$ ).



**Fig. 2** Dose–response relation for hERG inhibition. Detailed experimental procedure was described in Materials and methods section. Data represent mean  $\pm$  S.D. ( $n = 3$ )

## MDCK cell permeability

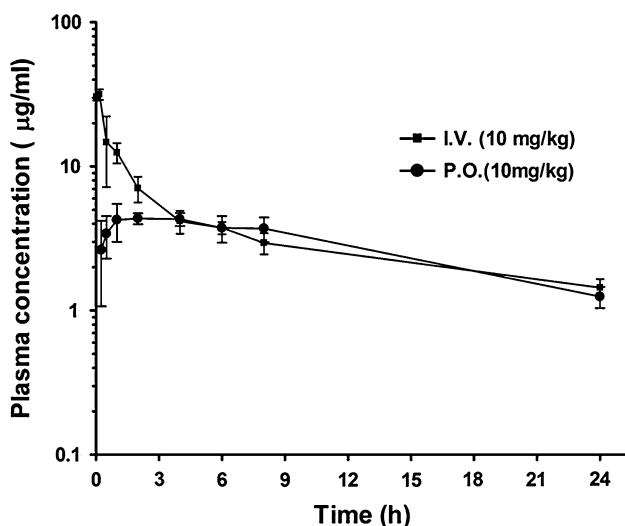
Intestinal absorption of drug is one of the key factors to determine its oral availability, efficacy, and toxicity. The epithelial cell monolayer system has been commonly used for the prediction of intestinal absorption. To assess the permeability of CX-4945, we used MDCK cell monolayer (apical to basal) system and found that CX-4945 exhibited a high apparent permeability coefficient ( $P_{app}$ ) value ( $10.7 \pm 0.99$  cm/s) at 50  $\mu$ M when metoprolol and atenolol exhibited  $12.9 \pm 0.07$  and  $0.07 \pm 0.01$  cm/s, respectively.

## Plasma protein binding

In general, the interaction between drug and plasma protein is a key factor that influences the efficiency of drugs. Interestingly, almost all amount of CX-4945 ( $98.8 \pm 1.0$  %) was existed as a binding form in rat blood plasma and there was no nonspecific binding against filter membranes.

## Pharmacokinetics of CX-4945 in rats

In vivo pharmacokinetics determines the absorption, metabolism, distribution, and excretion (commonly referred to as the ADME scheme) which are associated with the drug efficacy and toxicity of a specific drug (Ruiz-Garcia et al. 2008). The concentration of CX-4945 in rat blood plasma following intravenous or oral administration of CX-4945 at 10 mg/kg was shown in Fig. 3, and the pharmacokinetic parameters were listed in Table 2. CX-4945 was disappeared from plasma exhibiting long half-life



**Fig. 3** Plasma concentration–time plots after an intravenous and oral administration (10 mg/kg) in SD male rats ( $n = 3$ )

**Table 2** Pharmacokinetic parameters after an iv and oral administration of CX-4945 in SD male rats

Parameters	IV (10 mg/kg)	PO (10 mg/kg)
$T_{max}$ (h)	–	$1.50 \pm 0.71$
$C_{max}$ ( $\mu$ g/ml)	–	$4.74 \pm 1.09$
$T_{\lambda/2}$ (h)	$14.7 \pm 4.81$	$10.9 \pm 0.40$
$AUC_{0-24}$ ( $\mu$ g h/ml)	$90.0 \pm 11.9$	$71.2 \pm 18.3$
$AUC_{0-\infty}$ ( $\mu$ g h/ml)	$122 \pm 8.42$	$90.7 \pm 22.3$
CL (L/kg/h)	$0.08 \pm 0.01$	–
$V_{ss}$ (L/kg)	$1.39 \pm 0.58$	–
MRT (h)	$17.1 \pm 7.94$	$15.3 \pm 0.46$
F (%)		79.1 %

Data represent mean  $\pm$  S.D. ( $n = 3$ )

( $14.7 \pm 4.81$  h), steady-state volume of distribution ( $V_{ss}$ ) of  $1.39 \pm 0.58$  L/kg, and extremely low systematic clearance of  $0.08 \pm 0.01$  L/h/kg after intravenous administration. Similar to that of intravenous administrated result, CX-4945 showed a half-life of  $10.9 \pm 0.40$  h after oral administration. The oral bioavailability of CX-4945 was estimated to be  $\sim 79.1$  %. Thus, it might be considered that in vivo pharmacokinetic result is relevant to the MDCK cell permeability test.

## Conclusion

Since CX-4945 demonstrated an acceptable pharmacokinetics profiles such as long half-life and high oral bioavailability, and it exhibited the non-mutagenicity, non-genotoxicity and non-cardiac toxicity (Pierre et al. 2011), its successful clinical trial to give the hope to cancer patients is expected. Furthermore, the second generation of CK2 inhibitor, CX-8184, has been recently developed by Cylene pharmaceuticals. Thus, as well as the discussion of the results reported by Pierre et al. (Pierre et al. 2011) and our additional pharmacokinetic profiles of CX-4945, the comparison analysis of the pharmacokinetic profile of CX-4945 with that of CX-8184 in a further study would be helpful to understand its druggability for further development of CK2 inhibitors.

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