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Epothilone B-based 3-in-1 polymeric micelle for anticancer drug therapy

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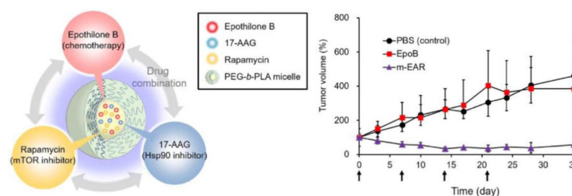
Abstract

Epothilones are microtubule inhibitors that are promising alternatives to paclitaxel due to enhanced anticancer efficacy. While epothilones are slightly more water soluble than paclitaxel and more active against paclitaxel-resistant cells, they still require formulation with Cremophor EL and/or cosolvents and drug resistance still limits therapeutic efficacy. In this report, we showed that the combinational treatment of epothilone B (EpoB), 17-N-allylamino-17-demethoxygeldanamycin (17-AAG, Hsp90 inhibitor), and rapamycin (mTOR inhibitor) displays strong anticancer activity *in vitro* and *in vivo*. To address the poor water solubility of this 3 drug-combination, they were co-loaded into poly(ethylene glycol)-*block*-poly(D,L-lactic acid) (PEG-*b*-PLA) micelles, and the 3-in-1 loaded PEG-*b*-PLA micelle (m-EAR) was characterized in terms of drug loading efficiency, particle size, release kinetics. The m-EAR achieved high levels of all three drugs in water; formed micelles with hydrodynamic diameters at ca. 30 nm and released the drugs in a sustained manner *in vitro* at rates slower than individually loaded PEG-*b*-PLA micelles. In A549-derived xenograft mice, m-EAR (2.0, 15.0, and 7.5 mg/kg) caused tumor regression after four weekly injections, whereas EpoB alone (2.0 mg/kg) was the same as control. No severe changes in body weight relative to PBS control were observed, attesting to the safety of m-EAR. Collectively, these results suggest that m-EAR provides a simple, but effective and safe EpoB-based combination nanomedicine for cancer therapy.

Graphical abstract



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Keywords

Epothilone B; polymeric micelle; non-small cell lung cancer; drug combination; multiple drug solubilization

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Epothilones have gained increasing attention due to much greater anticancer efficacy compared to paclitaxel (PTX). Epothilones induce cell cycle arrest at the G2-M transition and microtubule polymerization at nanomolar concentration, leading to cancer cell death (Goodin *et al.*, 2004). Compared to PTX, epothilones possess several advantages: Low susceptibility to drug efflux transporters, increased solubility and more manageable toxic profile in human patients (Bollag *et al.*, 1995; Bystricky and Chau, 2011). Bollag *et al.* has demonstrated that epothilone B (EpoB) has higher cytotoxicity and mitotic arrest at the G2-M transition against P-glycoprotein-expressing multi-drug resistant KBV-1 cells than PTX (Bollag *et al.*, 1995). Others have found that EpoB has strong *in vivo* anticancer activity at tolerated doses in several human cancer cell-derived xenograft models (Lin *et al.*, 2005; O'Reilly *et al.*, 2005; Pietras *et al.*, 2003; Rothermel *et al.*, 2003). However, researchers have concluded that single chemotherapy such as PTX and epothilones has reached a plateau in efficacy as a primary treatment modality (Bagnyukova *et al.*, 2010; LoPiccolo *et al.*, 2008). This is due to the growing recognition that cancer cells possess a capacity to activate compensatory signaling pathways, leading to drug resistance toward microtubule inhibitors (Hasenstein *et al.*, 2012).

To overcome drug resistance towards paclitaxel, the axis of mammalian target of rapamycin (mTOR) and heat shock protein 90 (Hsp90) has been widely investigated in pre-clinical and clinical studies. Rapamycin has improved the anticancer activity of PTX in MDA-MB-468 derived xenograft mice (Mondesire *et al.*, 2004). Combination therapy of rapamycin and 17-N-allylamino-17-demethoxygeldanamycin (17-AAG) has resulted in strong anticancer efficacy against breast cancer cells, blunting Akt activation due to mTOR inhibition (Roforth and Tan, 2008). 17-AAG has improved the anticancer efficacy of PTX in ovarian, lung and breast cancer xenograft models (Nguyen *et al.*, 2001; Sausville, 2001; Solit *et al.*, 2003). 17-AAG binds Hsp90 and inhibits client protein maturation, e.g. HER2 expression and Akt activation of cancer cells, increasing anticancer activity of PTX (Basso *et al.*, 2002; Schulte and Neckers, 1998). Furthermore, all three drugs have not only displayed cytotoxicity against cancer cells but also anti-angiogenesis ability, (Bocci *et al.*, 2002; Guba *et al.*, 2002; Kaur *et al.*, 2004). All these data suggest that combination therapy of 17-AAG and rapamycin might enhance anticancer efficacy of EpoB, achieved by the simultaneous inhibition of mTOR and Hsp90.

In this study, a 3-in-1 poly(ethylene glycol)-*block*-poly(D,L-lactic acid) (PEG-*b*-PLA) micelle containing EpoB, 17-AAG and rapamycin (m-EAR) has been prepared based on freeze-drying method (Fig. 1). Epothilones have required drug solubilization by cosolvents and/or Cremophor EL for intravenous injection or infusion for pre-clinical evaluation in human xenograft models and clinical trials. However, cosolvents and/or Cremophor EL are toxic; in the latter case, Cremophor EL causes life-threatening hypersensitivity reactions in ca. 3% of breast cancer patients despite pre-treatment with corticosteroid and antihistamine.

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Alternatively, PEG-*b*-PLA micelles act as a nanocarrier for poorly water-soluble anticancer agents, e.g. permitting 2-fold dose escalation of PTX relative to Taxol® (Kim et al., 2007). PEG-*b*-PLA micelles have been shown to solubilize sagophilone, an epothilone analog, and physicochemical properties of sagophilone-loaded PEG-*b*-PLA micelles have been characterized *in vitro* (Richter *et al.*, 2010).

Table 1 summarizes drug loading efficiency of PEG-*b*-PLA micelles: Single drug-loaded micelles (EpoB micelle: m-E, 17-AAG micelle: m-A and rapamycin micelle: m-R); two drug (EpoB/17-AAG micelle: m-EA, 17-AAG/rapamycin micelle: m-AR, 17-AAG/ rapamycin micelle: m-ER) or three drug-loaded micelles (m-EAR). PEG-*b*-PLA micelles increased the individual aqueous solubility of EpoB, 17-AAG and rapamycin (solubility in water: 25 μg/mL, 20 μg/mL, and 2.6 μg/mL, respectively) by 11-, 137- and 503-fold, resulting in drug level at 0.28, 2.74 and 1.31 mg/mL, respectively (Table 1) (Kaur *et al.*, 2004; Simamora *et al.*, 2001). Notably, % drug loading efficiency for each drug in 2-in-1 and m-EAR was similar with that of single 1-in-1 drug-loaded micelles (70~90%), showing that PEG-*b*-PLA micelles have capacity for not only one drug but also two or three drugs. The results on m-EAR are consistent with our recent work on PEG-*b*-PLA micelles that has proven that they can solubilize not only one drug but also two or three drugs as a multi-drug nanocarrier, including PTX, 17-AAG and rapamycin (Shin et al., 2009; Tomoda et al., 2016). The average hydrodynamic diameters of PEG-*b*-PLA micelles was 25~30 nm, regardless of drug loading efficiency (Fig. S1 and Table S1), and PDI values were < 0.1, indicating low polydispersity. Based on particle size analysis by DLS, all drug-loaded PEG-*b*-PLA micelles were stable for five days at ambient temperature (Fig. S2). However, purple precipitate was observed only in m-A after five days, indicating the loss of physical stability of 17-AAG (Fig. S3). In contrast, m-A prepared by a polymer film hydration method had higher 17-AAG loading and was more stable (Shin et al., 2011), indicating that polymer film hydration method is better for m-A between two methods. The molecular rationale for differences for 17-AAG requires further study. No precipitation was observed in m-EA, m-AR, and m-EAR.

In vitro release of EpoB from PEG-*b*-PLA micelles was rapid with $t_{1/2}$ =2.28 hours, assuming first-order kinetics (Figure 2). While the $t_{1/2}$ values for 17-AAG and rapamycin were 9.42 and 19.1 hours, respectively, increasing with $\log P_{oil/water}$ value. Notably, *in vitro* drug release for m-EAR was noticeably slower than 1-in-1 drug-loaded PEG-*b*-PLA micelles, having $t_{1/2}$ values for EpoB, 17-AAG and rapamycin at 5.98, 18.0 and 28.2 hours, respectively (Table S2). This may reflect intermolecular drug interaction in the core of PEG-*b*-PLA micelles that allows slower drug release. Alternatively, location of co-loaded drugs in PEG-*b*-PLA micelles may impact drug release. ^{1}H NMR analysis of m-EAR might give insights into drug location and possibly drug-drug interactions in PEG-*b*-PLA micelles (Catenacci et al., 2014). One limitation of this *in vitro* study is noteworthy: It was done above the CMC of PEG-*b*-PLA micelles and reflects diffusional release, but not the contribution due to micelle disassembly, owing to dilution below the CMC after injection in blood. The rapid *in vitro* release of EpoB indicates that PEG-*b*-PLA micelles will act primarily as a non-toxic solubilizing agent but not a long-circulating nanocarrier for EpoB; however this hypothesis requires pharmacokinetic validation.

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Table 2 summarizes the *in vitro* cytotoxicity of drug(s) against human A549 non-small cell lung cancer cells. EpoB has indeed more potent cytotoxicity against cancer cells than PTX (IC_{50} = 0.17 *versus* 3.16 nM), and this result is consistent with previous studies (Altmann *et al.*, 2000; Bollag *et al.*, 1995). After treatment of A549 cells with drug combinations, drug interaction (synergistic, additive or antagonistic) between EpoB, 17-AAG and rapamycin was evaluated using combination index (CI) analysis. CI values of drug combinations were calculated based on inhibitory concentration ($IC_{35,\ 50,\ \text{and}\ 65}$) values of individual drugs and their combinations. CI values of two drug combinations were variable, showing synergy for EpoB with rapamycin, but additivity or slight antagonism for EpoB with 17-AAG. The CI value of the 3-drug combination also showed synergy, additivity or slight antagonism, depending on the fraction of A549 cell affected. In this case, the IC_{50} value was 0.29 nM, approaching the remarkably low value of EpoB.

Fig. 3 shows % colony formed by A549 non-small lung cancer cells in the presence or absence of anticancer agents. EpoB (1 nM), 17-AAG (1 nM) and rapamycin (1 nM) as single agents caused a slight reduction in colonies, with rapamycin causing the highest reduction at ca. 40%. Two drug combinations were more effective, noting the low number of colonies for EpoB and 17-AAG, ca. 15%. Remarkably, no colonies were observed after treatment of EpoB/17-AAG/rapamycin (1/1/1 nM), indicating that three drug combination therapy is highly effective in cancer cell reproductive death.

Fig. 4 shows the antitumor efficacy of m-EAR *versus* EpoB in an A549 xenograft model. Treatment of female athymic nude mice started after tumors were palpable, ca, 50-100 mm^3. At 2.0 mg/kg, EpoB was equivalent to the control (PBS) with tumor growth increasing steadily even during weekly treatment. In contrast, four weekly injections of m-EAR at 2.0, 15.0, and 7.5 mg/kg for EpoB, 17-AAG, and rapamycin, respectively, caused tumor regression without tumor regrowth at the termination of treatment. Moreover, despite concurrent three drug injection, m-EAR caused no discernible change in body weight relative to EpoB alone, indicating an absence of overlapped toxicity. This result is due to sustained release of drugs, supporting at least partially a role of PEG-*b*-PLA micelles in reducing toxicity for m-EAR.

In summary, a 3-in-1 poly(ethylene glycol)-*block*-poly(D,L-lactic acid) (PEG-*b*-PLA) micelle containing EpoB, 17-AAG and rapamycin has been prepared, characterized and shown to be highly effective against A549 non-small cell cancer cells *in vitro* and *in vivo*. Given the high antitumor efficacy over EpoB and low acute toxicity, m-EAR deserves further research in a pre-clinical setting, aiming for clinical evaluation of m-EAR for cancer treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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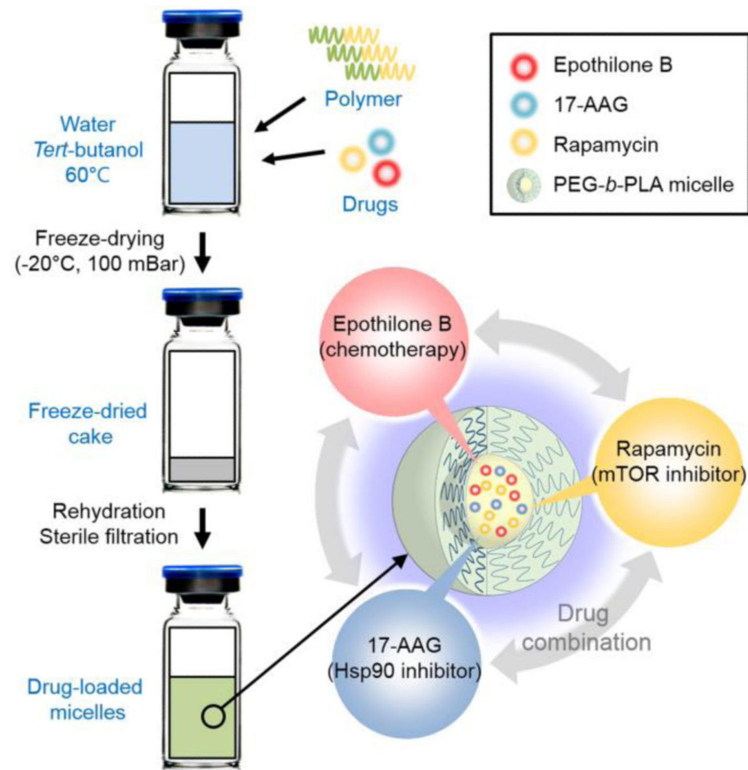

Fig. 1.
Schematic representation of 3-in-1 PEG-*b*-PLA micelle preparation.

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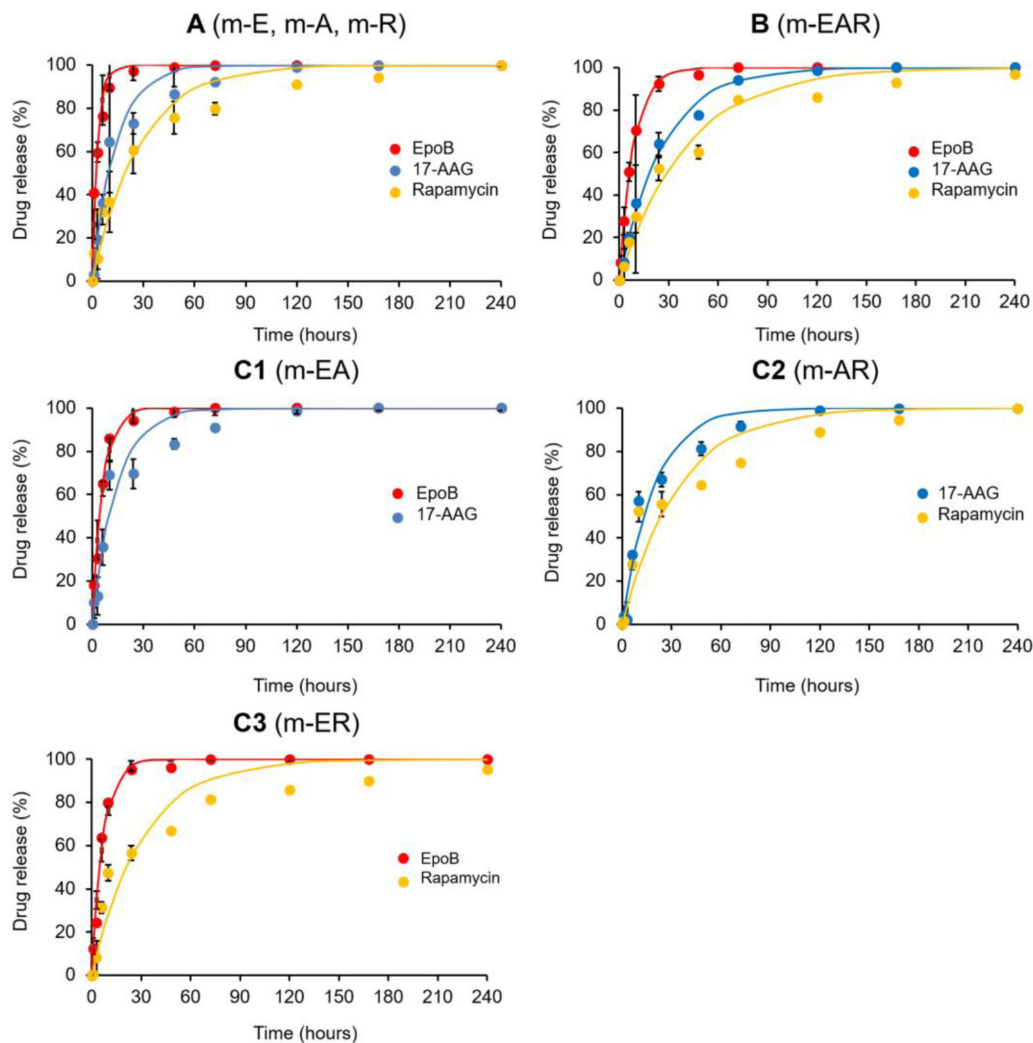

Fig. 2.

In vitro drug release profiles of various micellar formulations containing one drug only (A), three drug combination (B) and two drug combinations (C). All measurements were performed three times.

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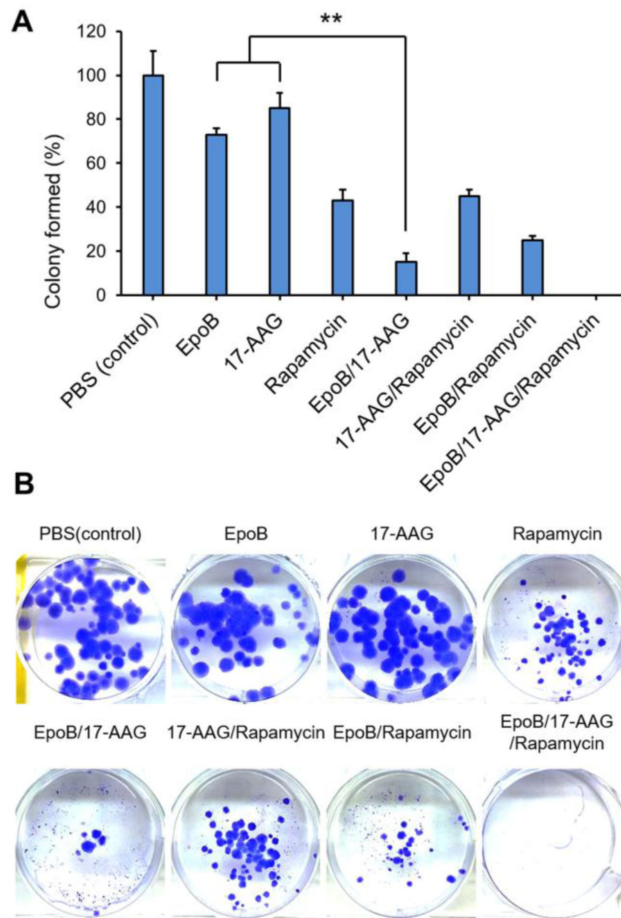

Fig. 3.

(A) *In vitro* clonogenic assay of various formulations. Colony formation in each well after treatment of EpoB (1 nM), 17-AAG (1 nM), and rapamycin (1 nM) or their two or three combinations for two weeks was quantified. (B) Representative photographs of the colonies in each well after drug treatment (n=3).

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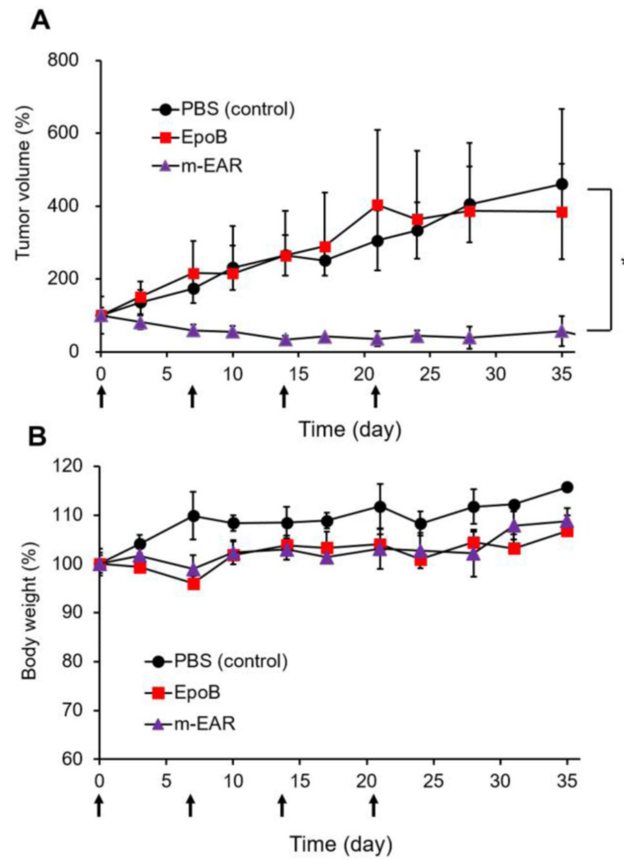

Fig. 4.

Antitumor effect of various formulations in A549 xenograft nude mice. Mice were treated weekly (indicated by arrows in the figure) and tumor growth (A) and body weight changes (B) were monitored periodically. EpoB (2.0 mg/kg). 17-AAG (15 mg/kg). Rapamycin (7.5 mg/kg) (n=5).

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Table 1

In vitro characterization of PEG-*b*-PLA micelles (n=3).

Type	Micelles	Polymer (mg/mL)	Initial level of drug(s) (mg/mL)	Drug loading efficiency (%)	Individual drug level (mg/mL)	Total drug level (mg/mL)
1-in-1	m-E	100	0.4	70.6±3.2	0.282±0.013	0.282±0.013
	m-A	100	3	91.5±4.7	2.744±0.141	2.744±0.141
	m-R	100	1.5	87.6±5.1	1.314±0.077	1.314±0.077
2-in-1	m-EA	100	0.4/3	83.7±4.9 82.7±4.0	0.335±0.019 2.482±0.121	2.817±0.141
	m-AR	100	3/1.5	82.3±3.9 83.9±3.6	2.470±0.118 1.258±0.054	3.728±0.172
	m- ER	100	0.4/1.5	73.8±2.8 77.8±4.1	0.295±0.011 1.167±0.062	1.462±0.073
3-in-1	m-EAR	100	0.4/3/1.5	88.6±0.5 83.0±0.7 82.2±0.7	0.354±0.002 2.491±0.021 1.232±0.010	4.078±0.033

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Table 2

Evaluation of drug interaction: CI analysis of 2- and 3-drug combinations (n=3).

Drug combination (molar ratio)	PTX	EpoB	17-AAG	Rapamycin	EpoB /17-AAG (1:1)	17-AAG /Rapamycin (1:1)	EpoB /Rapamycin (1:1)	EpoB /17-AAG /Rapamycin (1:1:1)
IC_{35} (nM)	1.98±0.03	0.10±0.00	8.52±0.24	0.07±0.01	0.27±0.00	0.12±0.00	0.03±0.00	0.09±0.00
IC_{50} (nM)	3.16±0.06	0.17±0.00	19.95±0.16	0.25±0.03	0.52±0.01	0.35±0.00	0.09±0.00	0.29±0.00
IC_{65} (nM)	5.59±0.24	0.33±0.01	62.97±1.71	2.32±0.62	1.28±0.04	1.46±0.11	0.30±0.01	1.24±0.08
CI_{35}	-	-	-	-	1.37	0.81	0.41	0.70
CI_{50}	-	-	-	-	1.54	0.70	0.45	0.97
CI_{65}	-	-	-	-	1.97	0.33	0.53	1.45