Pharmacodynamic Modeling Identifies Synergistic Interaction Between Chloroquine and Trastuzumab in Refractory HER2-positive Breast Cancer Cells

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Abstract. *Background/Aim: Despite improvements in HER2-positive breast cancer (BC) patients' outcomes with trastuzumab, the occurrence of intrinsic or acquired resistance presents a clinical challenge. Here, we quantitatively assess the combinatorial effects of chloroquine, an autophagy inhibitor, with trastuzumab on JIMT-1 cells, a HER2-positive BC cell-line primarily resistant to trastuzumab. Materials and Methods: The temporal changes in JIMT-1 cellular viability were assessed using the CCK-8 kit, where JIMT-1 cells were exposed for 72-h to trastuzumab (0.007-17.19 μM) or chloroquine (5-50 μM) as single-agents, in combination (trastuzumab: 0.007-0.688 μM; chloroquine: 5-15 μM), or control (no drug). Concentration-response relationships were built for each treatment arm to determine drugs' concentrations inducing 50% of cell-killing (IC50). Cellular pharmacodynamic models were built to characterize the time-trajectory of JIMT-1 cellular viability under each treatment arm. The nature of trastuzumab and chloroquine interaction was quantified by estimating the interaction parameter (Ψ). Results: The IC50 were estimated at 19.7 and 24.4 μM for trastuzumab and chloroquine. The maximum killing effect was about thrice higher for chloroquine than trastuzumab (0.0405 vs. 0.0125 h^{-1}), validating chloroquine's superior anti-cancer effect on JIMT-1 cells compared to trastuzumab. The time-delay for chloroquine cell-killing was twice longer than that for trastuzumab (17.7 vs. 7 h), suggesting a chloroquine time-dependent anti-cancer effect. The Ψ was determined at 0.529 (Ψ<1), indicating a synergistic interaction. Conclusion: This proof-of-concept study on JIMT-1 cells identified chloroquine and trastuzumab synergistic interaction, warranting further in vivo investigations.*

The human epidermal growth factor receptor-2 (HER2) is overexpressed in approximately 20-30% of breast cancer (BC) cases. HER2 positive BC cells have a tendency to proliferate and spread aggressively leading to higher recurrence and mortality rates (1, 2). The HER2 receptor belongs to the epidermal growth factor (EGF) family of receptor tyrosine kinases; although this receptor does not bind to ligands, it dimerizes with HER1, HER3 and HER4 to activate downstream signaling cascades that control biological processes such as cell growth and survival (3).

Trastuzumab (TZB) is a recombinant humanized monoclonal antibody that targets the HER2 receptor. Once TZM binds to the extracellular domain of the HER2 receptor, multiple mechanisms are triggered including the prevention of HER2-receptor dimerization, shedding of the extracellular domain, immune activation, as well as inhibition of its downstream intracellular tyrosine kinases (4). Despite the success of HER2 targeted therapies, such as TZB, the occurrence of intrinsic and acquired resistance has been reported. Some mechanisms of TZM resistance include the truncation of the HER2 receptor, increased signaling from other HER receptors such as HER3 or EGFR, increased signaling by the insulin-like growth factor receptor (IGF-1), loss of the AKT regulator phosphatase and TENsin homolog (PTEN), and/or lack of immune response (5).

Figure 1. *Concentration-response curves for chloroquine (A) and trastuzumab (B). Black circles represent observed data and green smooth lines represent model fittings.*

In addition, induction of autophagy, a cellular survival mechanism activated in response to metabolic stress, hypoxia, or chemotherapy-induced cell death, has also been linked to TZB resistance. Briefly, during autophagy, autophagosomes are formed and engulf cytoplasm/cytoplasmic organelles. Then the autophagosomes fuse with lysosomes to degrade the contents of the autophagosome and make amino acids and other by-products available for metabolism and building of macromolecules (6). In a publication by Vazquez-Martin *et al.*, it was reported that TZB resistant BC cells displayed increased cellular levels of the LC3-II protein, which is correlated with increased autophagosome numbers (7, 8). The anti-malarial chloroquine (CQ) has been found to inhibit autophagy by impairing autophagosome fusion with lysosomes and increasing the pH inside lysosomes to inhibit enzymatic function. CQ also induces disorganization of the golgi and endo-lysosomal systems, which may further contribute to fusion impairment. As a result, adding CQ to TZB therapy may decrease resistance to HER2 therapy in TZM-treated breast cancer (7, 9).

In this work, we sought to test *in vitro* the hypothesis that inhibition of autophagy with a common autophagy inhibitor such as CQ may be a valuable mechanism to reverse the resistance to TZM therapy in refractory HER2-positive BC. We examined the anti-cancer effects of TZM and CQ as single agents and in combination on JIMT-1 cells, a HER2 positive BC cell-line primarily resistant to TZM, and quantitatively assessed the nature of their drug-drug interaction. Our findings of synergistic combinatorial killing effects of TZM and CQ on JIMT-1 cells support the aforementioned hypothesis; however, an *in vivo* confirmatory study is warranted.

Materials and Methods

Drugs and reagents. CQ was purchased from Selleck chemical (Houston, TX, USA) and TZB was acquired from the pharmacy of the University of Florida (Gainesville, FL, USA). Dulbecco's Modified Eagle's Medium (DMEM), Penicillin/Streptomycin, and Phosphate Buffered Saline (PBS) were purchased from Hyclone, GE Healthcare Biosciences (Chicago, IL, USA). MEM non-essential amino acids, molecular biology grade water and 0.25% trypsin/2.21 mM EDTA were acquired from Corning (Corning, NY, USA). Fetal bovine serum (FBS) and Cell Counting kit-8 (CCK-8) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

CQ was dissolved in molecular biology grade water to make a 100 mM stock. CQ stocks were stored at –80°C and TZM was stored at 4°C, per manufacturer instructions. Fresh serial dilutions of drugs were prepared prior to experiments.

Cell culture. JIMT-1 cells, a TZM-resistant HER2+ cell line, were acquired from AddexBio (San Diego, CA, USA) and maintained in DMEM supplemented with 10% FBS, 1% sodium bicarbonate, 1% MEM non-essential amino acids, and 1% Penicillin/Streptomycin. Cells were incubated at 37°C in a humidified atmosphere with 5% CO2 and passaged once confluent with 0.25% trypsin/2.21 nM EDTA.

In vitro cytotoxicity. JIMT-1 cells were seeded at a density of 3,000 cells/100 µl/well in a 96-well plate and incubated for at least 24 h to ensure adhesion. Concentration-response relationships were generated by exposing JIMT-1 cells to either single agent TZM (0.007-51.54 µM) or CQ (0.5-1000 µM) over 72 h. Experiments were performed in quadruplicates and compared against vehicle control (cell culture media). Cell viability was determined by incubating the cells in CCK-8 solution (10 µl/well of a 96-well plate) for 1.5 h and measuring absorbance at 450 nm using a microplate spectrophotometer (Biotek, Winooski, VT, USA). In subsequent experiments, JIMT-1 cells were exposed to TZM (0.007-17.19 µM) or CQ (5-50 µM) single agents or combinations of these (TZB: 0.007-0.688 µM; CQ: 5-15 µM) over a 72-h time course. Cellular viability was measured using the CCK-8 assay as described above.

Mathematical modeling.
Concentration-response relationships. The maximal inhibitory effects (Imax) for CQ and TZB and their corresponding concentrations leading to 50% of Imax (IC$_{50}$) were estimated at 72 h by modeling their respective concentration-response curves with an inhibitory Hill function (10) such as:

$$R = R_0 \cdot \left(1 - \frac{Imax \cdot C^{\gamma}}{IC_{50}^{\gamma} + C^{\gamma}}\right) \qquad (1)$$

where, R is the response to treatment (% cell viability), R0 is the baseline response (% viability under control conditions), Imax is the maximal effect, C is the concentration of drugs, IC50 is the drug concentration corresponding to 50% of Imax, and γ is the Hill coefficient. All mathematical modeling was performed with Monolix version 2016R1 (Antony, France: Lixoft SAS, 2016).

Time course cellular response pharmacodynamic models. JIMT-1 cellular response following exposure to single agent CQ, TZB, or combinations of CQ and TZB were measured for 0, 24, 48 and 72 h.

Cell growth for the control arm was described with an exponential growth function:

$$\frac{dR}{dt} = k_g \cdot R; \quad \text{R(0)=R}_0 \qquad (2)$$

where k_g represents the first order JIMT-1 growth rate constant, and R represents cellular response.

Single agent pharmacodynamic models. Pharmacodynamic (PD) models to characterize the cellular response profiles of CQ and TZB required two and four transit compartments (11) to adequately capture the observed delays between drug exposure and declines in cell viability as a result of the stimulation of cell death. The respective equations are described below:

$$\frac{dK1_{CQ}}{dt} = \left(\frac{1}{\tau_{CQ}}\right) \cdot \left(\left(\frac{S_{max_{CQ}} \cdot C_{CQ}^{\gamma_1}}{C_{CQ}^{\gamma_1} + SC_{50_{CQ}}^{\gamma_1}}\right) - K1_{CQ}\right);$$
$$\text{K1}_{CQ}\text{(0)=0} \qquad (3)$$

$$\frac{dK2_{CQ}}{dt} = \left(\frac{1}{\tau_{CQ}}\right) \cdot \left(K1_{CQ} - K2_{CQ}\right); \quad \text{K2}_{CQ}\text{(0)=0} \quad (4)$$

$$\frac{dR_{CQ}}{dt} = k_g \cdot R_{CQ} - K2_{CQ} \cdot R_{CQ}; \quad \text{R}_{CQ}\text{(0)=0} \quad (5)$$

$$\frac{dK1_{TZB}}{dt} = \left(\frac{1}{\tau_{TZB}}\right) \cdot \left(\left(\frac{S_{max_{TZB}} \cdot C_{TZB}^{\gamma_2}}{C_{TZB}^{\gamma_2} + SC_{50_{TZB}}^{\gamma_2}}\right) - K1_{TZB}\right);$$
$$\text{K1}_{TZB}\text{(0)=0} \qquad (6)$$

$$\frac{dK2_{TZB}}{dt} = \left(\frac{1}{\tau_{TZB}}\right) \cdot (K1_{TZB} - K2_{TZB}); \quad \text{K2}_{TZB}\text{(0)=0} \quad (7)$$

$$\frac{dK3_{TZB}}{dt} = \left(\frac{1}{\tau_{TZB}}\right) \cdot (K2_{TZB} - K3_{TZB}); \quad \text{K3}_{TZB}\text{(0)=0} \quad (8)$$

$$\frac{dK4_{TZB}}{dt} = \left(\frac{1}{\tau_{TZB}}\right) \cdot (K3_{TZB} - K4_{TZB}); \quad \text{K4}_{TZB}\text{(0)=0} \quad (9)$$

$$\frac{dR_{TZB}}{dt} = k_g \cdot R_{TZB} - K4_{TZB} \cdot R_{TZB}; \quad \text{R}_{TZB}\text{(0)=0} \quad (10)$$

Where τ represents mean transit time, K1-K4 represent the transit compartments, C represents drug concentrations, S_{max} represents the

maximal value of the stimulatory cell death constant, SC$_{50}$ represents the concentration corresponding to half of S$_{max}$, and γ represents the hill coefficient.

Chloroquine and trastuzumab pharmacodynamic model. Since TZB and CQ both exhibited cytotoxic effects over JIMT-1 cells, an interaction parameter, psi (Ψ), was applied to equation 3, to determine the nature of the CQ-TZM drug-drug interaction. Psi values below 1 indicate a synergistic interaction, values above 1 indicate an antagonistic interaction, while values equivalent to 1 indicate an additive interaction. The equations are described as follows:

$$\frac{dK1_{CQ}}{dt} = \left(\frac{1}{\tau_{CQ}}\right) \cdot \left(\left(\frac{S_{max_{CQ}} \cdot C_{CQ}^{\gamma_1}}{C_{CQ}^{\gamma_1} + \left(\Psi \cdot SC_{50_{CQ}}^{\gamma_1}\right)}\right) - K1_{CQ}\right);$$
$$\text{K1}_{CQ}\text{(0)=0} \qquad (11)$$

$$\frac{dK2_{CQ}}{dt} = \left(\frac{1}{\tau_{CQ}}\right) \cdot \left(K1_{CQ} - K2_{CQ}\right); \quad \text{K2}_{CQ}\text{(0)=0} \quad (12)$$

$$\frac{dK1_{TZB}}{dt} = \left(\frac{1}{\tau_{TZB}}\right) \cdot \left(\left(\frac{S_{max_{TZB}} \cdot C_{TZB}^{\gamma_2}}{C_{TZB}^{\gamma_2} + SC_{50_{TZB}}^{\gamma_2}}\right) - K1_{TZB}\right);$$
$$\text{K1}_{TZB}\text{(0)=0} \qquad (13)$$

$$\frac{dK2_{TZB}}{dt} = \left(\frac{1}{\tau_{TZB}}\right) \cdot (K1_{TZB} - K2_{TZB});$$
$$\text{K2}_{TZB}\text{(0)=0} \qquad (14)$$

$$\frac{dK3_{TZB}}{dt} = \left(\frac{1}{\tau_{TZB}}\right) \cdot (K2_{TZB} - K3_{TZB});$$
$$\text{K3}_{TZB}\text{(0)=0} \qquad (15)$$

$$\frac{dK4_{TZB}}{dt} = \left(\frac{1}{\tau_{TZB}}\right) \cdot (K3_{TZB} - K4_{TZB});$$
$$(16)$$

$$\frac{dR_{CT}}{dt} = k_g \cdot R_{CT} - \left(K2_{CQ} + K4_{TZB}\right) \cdot R_{CT};$$
$$\text{R}_{CT}\text{(0)=0} \qquad (17)$$

Statistical analysis. To determine if the reductions in cellular viability following exposure to CQ+TZB combinations were significantly different from those observed post-CQ single agent exposure, statistical analyses of relative cell viabilities at 24, 48 and 72 h were performed *via* one-way ANOVA followed by Dunnett's multiple comparison test, with an α of 0.05. Statistical analyses were performed with GraphPad Prism ver. 5 (GraphPad Software, La Jolla, CA, USA).

Results

Concentration-response relationships. The model fits of the measured percent cell viability for JIMT-1 cells *vs*. a range of concentrations of TZB and CQ are depicted in Figure 1 and the corresponding model fitted parameters are summarized in
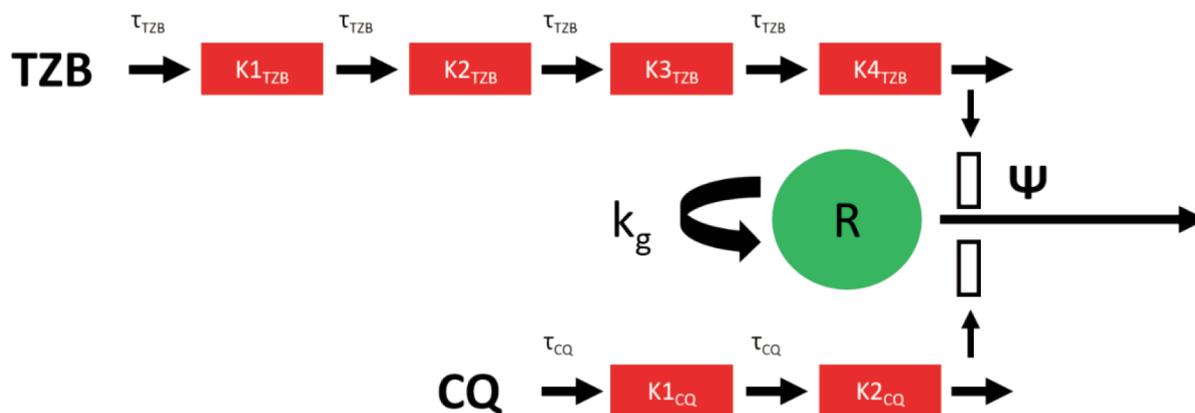
Figure 2. *Model schematic for chloroquine (CQ) and trastuzumab (TZB) singe agents and combinations. K1-K4 represent transit compartments required to account for delays in cytotoxicity, while τ_{CQ} and τ_{TZB} represent the transit times for CQ and TZB. k_g represents the first-order growth rate constant for JIMT-1 cells, R represents cellular response, and Ψ represents an interaction parameter for stimulation of cell death when CQ and TZB are combined. The open rectangles represent stimulation of cell death.*

Table I. The observed data were characterized well with inhibitory Hill functions, with all model parameters estimated with reasonable precision. The IC_{50}, or concentration required for 50% of maximal drug effect, for CQ and TZB were 24.4 and 19.7 μM, respectively. For both drugs the highest tested concentrations reduced cell viability to approximately 0%, thus I_{max} (maximal effect) was fixed to 1.

Cellular response time course. Cellular response pharmacodynamic (PD) models were built to characterize JIMT-1 cellular viability following exposure to CQ and TZM single agents and combinations over a 72-h time course. The schematic summarizing the PD models for the single agents and combinations is depicted in Figure 2, graphs of cellular viability for all treatment arms are depicted in Figure 3, and the model parameters are summarized in Table II.

Both CQ and TZB stimulated JIMT-1 cell death, with 2 transit compartments required to capture the observed delays in cytotoxicity for CQ and 4 compartments required for TZB. The estimated maximal value of the stimulatory death constant (S_{max}) for CQ was 0.0405 h^{-1}, with an SC_{50} (concentration required to achieve half of S_{max}) of 26.3 μM, while the estimated S_{max} and SC_{50} for TZB were 0.0124 h^{-1} and 12.6 μM. As both CQ and TZB exerted cytotoxic effects over JIMT-1 cells, the interaction parameter (Ψ) was estimated as 0.529±0.014, indicating a synergistic interaction.

Statistical analysis. Finally, to determine if the observed cellular viabilities between CQ single agent treated cells were significantly different from CQ+TZB treated cells, relative cell viabilities for the 24-, 48- and 72-h time points were analyzed by one-way Analysis of Variance (ANOVA), followed by Dunnett's multiple comparison test. Figure 4

Table I. *Concentration-response curve parameter estimates and percent relative standard error (% RSE) for chloroquine (CQ) and (trastuzumab).*

Parameters (unit)	Definition	Parameter estimates (% RSE)	
		CQ	TZB
R_0 (%)	Baseline cell viability	98.2 (1)	100 (Fixed)
IC_{50} (μM)	Drug concentration for 50% of maximal effect	24.4 (4)	19.7 (4)
Imax	Maximal effect	1 (Fixed)	1 (Fixed)
γ	Hill coefficient	1.7 (6)	4.3 (12)

depicts the average relative cell viabilities±standard deviation (SD) and Table III summarizes the ANOVA results. Generally, this analysis demonstrated that cell viability of the combination treatment arms was significantly different from that of single agent arm for the 24 and 72-h time points.

Discussion

HER2-positive BC represents 25% of all BC subtypes and is an aggressive BC that is associated with poor treatment outcomes and low patients' survival rates (1, 2). In this work, we designed an *in vitro* proof-of-concept study aiming to test the hypothesis that CQ, an autophagy inhibitor, may alleviate resistance to TZM in HER2-positive BC that is refractory to TZM. To this end, we performed *in vitro* experiments on JIMT-1 cells, a HER2-positive BC cell-line that is primarily resistant to TZM. We quantified JIMT-1 cells viability under single and combinatorial effects of CQ with TZM at
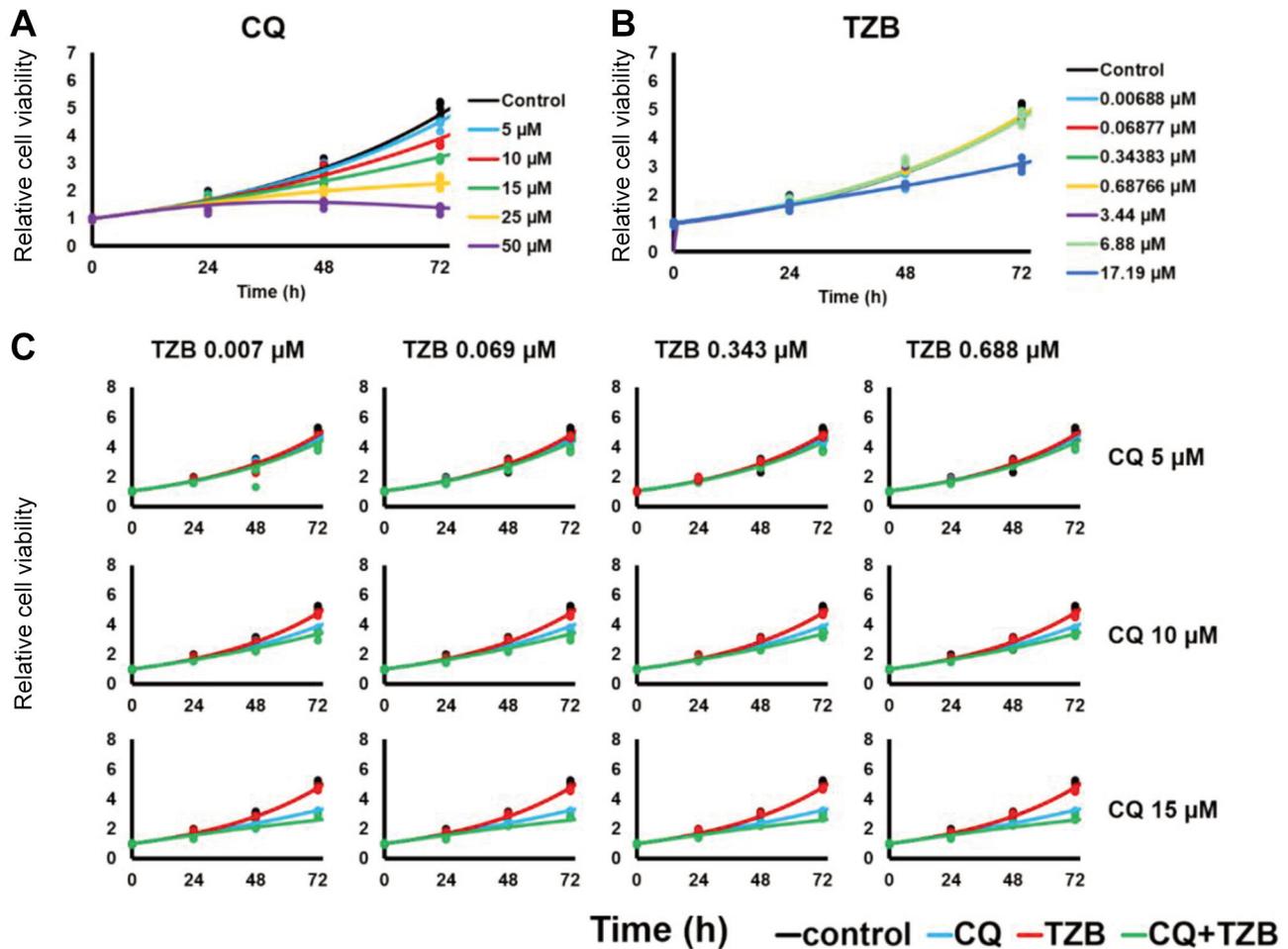
Figure 3. *Model fittings for 0 to 72-h cellular viability profiles of JIMT-1 cells following exposure to single agent chloroquine (CQ) (A), single agent trastuzumab (TZM) (B), and CQ+TZB combinations (C). Colored circles represent observed data (n=4) and smooth colored lines represent model fittings of the corresponding-colored observed data, where each color corresponds to a treatment arm.*

increasing concentrations of each drug and exposure times. We also characterized mathematically these concentration- and time-effect relationships.

The concentration-response relationships in JIMT-1 cells from single agents CQ and TZM were quantified pharmacologically and the IC_{50} of each drug was determined. Our results confirm that JIMT-1 cells are indeed resistant to TZM, since the IC_{50} was estimated at 19.7 μM, which is equivalent to 2,867 μg/ml. This concentration is approximately 23-fold higher than the reported clinical serum concentration at steady-state of 123 μg/ml (12). Besides the fact that JIMT-1 cells are inherently resistant to TZM, one other possible interpretation to the lower concentration of TZM *in vivo* compared to our estimated *in vitro* IC_{50} is that in the tumor micro-environment, the TZM main mechanism of action of TZM is known to be through antibody-dependent cell-mediated cytotoxicity (ADCC). The ADCC is an adaptive immune response mediated mainly by the natural killer (NK) cells that bind the Fc portion of TZM, triggering the lysis of tumor cells. The absence of NK cells in the *in vitro* cell culture setting and, hence, lack of ADCC effect by TZM led to the need for a larger TZM concentration to achieve a desired level of JIMT-1 cell killing.

Despite the unclear clinical utility of CQ in cancer therapy, our findings agree with other published studies conducted *in vitro* on various cancer cell lines including lung (13), breast (14), bladder (15) and colon cancer (16). In these studies, CQ has been reported to induce apoptosis at concentrations ranging from 25 to 128 μM. Notwithstanding that in adults, CQ toxicity is dose dependent. It was shown that the ingestion of CQ at doses between 2 to 4 g may cause neurological symptoms corresponding to blood concentrations between 2.5 and 5 mg/l (4.84-9.69 μM), while CQ blood concentrations above 5 mg/l have been associated

Table II. *Cellular response model fitted parameters and relative standard error (% RSE).*

Parameter (unit)	Description	Estimate	%RSE
R_0	Baseline cell viability	1	Fixed
k_g (h^{-1})	First-order JIMT-1 growth rate constant	0.0217	0.35
S_{maxCQ} (h^{-1})	Maximal value of the stimulatory death constant for CQ	0.0405	17
SC_{50CQ} (μM)	Concentration of CQ where half of S_{max} is achieved	26.3	11
γ_1	Hill coefficient for CQ	1.97	8
τ_{CQ} (h)	Transit time for CQ	17.5	18
S_{maxTZB} (h^{-1})	Maximal value of the stimulatory death constant for TZM	0.0124	29
SC_{50TZB} (μM)	Concentration of TZM where half of S_{max} is achieved	12.6	27
γ_2	Hill coefficient for TZM	5	Fixed
τ_{TZB} (h)	Transit time for TZM	7.4	25
Ψ	Interaction parameter psi	0.529	3

Table III. *Analysis of Variance (ANOVA) and Dunnett's multiple comparison test for comparison of reductions in cellular viability following exposure to chloroquine and trastuzumab (CQ+TZB) combinations with CQ at 24, 48 and 72 h post-exposure.*

Treatment arm		Significance		
		24 h	48 h	72 h
CQ 5 μM	ANOVA:	$p<0.05$ (S)	$p>0.05$ (NS)	$p<0.05$ (S)
	Dunnett's multiple comparison test			
	CQ 5 μM+TZB 0.007 μM	$p<0.05$ (S)	N/A	$p<0.05$ (S)
	CQ 5 μM+TZB 0.069 μM	$p<0.05$ (S)	N/A	$p<0.05$ (S)
	CQ 5 μM+TZB 0.343 μM	$p>0.05$ (NS)	N/A	$p<0.05$ (S)
	CQ 5 μM+TZB 0.688 μM	$p<0.05$ (S)	N/A	$p<0.05$ (S)
CQ 10 μM	ANOVA:	$p<0.05$ (S)	$p<0.05$ (S)	$p<0.05$ (S)
	Dunnett's multiple comparison test			
	CQ 10 μM+TZB 0.007 μM	$p<0.05$ (S)	$p<0.05$ (S)	$p<0.05$ (S)
	CQ 10 μM+TZB 0.069 μM	$p<0.05$ (S)	$p<0.05$ (S)	$p<0.05$ (S)
	CQ 10 μM+TZB 0.343 μM	$p<0.05$ (S)	$p<0.05$ (S)	$p<0.05$ (S)
	CQ 10 μM+TZB 0.688 μM	$p<0.05$ (S)	$p<0.05$ (S)	$p<0.05$ (S)
CQ 15 μM	ANOVA:	$p<0.05$ (S)	$p>0.05$ (NS)	$p<0.05$ (S)
	Dunnett's multiple comparison test			
	CQ 15 μM+TZB 0.007 μM	$p<0.05$ (S)	N/A	$p>0.05$ (NS)
	CQ 15 μM+TZB 0.069 μM	$p<0.05$ (S)	N/A	$p<0.05$ (S)
	CQ 15 μM+TZB 0.343 μM	$p<0.05$ (S)	N/A	$p<0.05$ (S)
	CQ 15 μM+TZB 0.688 μM	$p<0.05$ (S)	N/A	$p<0.05$ (S)

N/A: Not applicable; NS: statistically not significant; S: statistically significant.

with serious toxicity (17). While the CQ estimated IC$_{50}$ from our experimental data on JIMT-1 cells is close to the lower bound of its concentration interval in various *in vitro* cancer cells (24.4 *vs.* 25 μM), it is calculated to be ~2.5-fold the *in vivo* serum concentration causing serious safety concerns (24.4 *vs.* 9.69 μM). Hence, based on the supra-therapeutic estimated *in vitro* IC$_{50}$ for CQ and TZM in JIMT-1 cells, we conclude that as single-agents CQ and TZM require above therapeutic blood concentrations to be efficacious at killing HER2-positive BC cells refractory to TZM.

In subsequent experiments, we exposed JIMT-1 cells to a range of CQ and TZB concentrations, as single-agents and in combination over a 72-h time course. Cell-based PD models were built to characterize the time course data and to determine the nature of their drug-drug interaction. Both CQ and TZB stimulated JIMT-1 cell death, with an estimated interaction parameter Ψ of 0.529±0.014, indicating synergism ($\Psi<1$) between these agents. Upon visual inspection of the cellular response plots, it appeared that JIMT-1 cellular viability under the combination treatment was slightly lower than the JIMT-1 cellular viability under the treatment with single agent CQ. To determine if these differences were statistically significant, the relative cell viability at 24-, 48-, and 72-h time points was analyzed by one-way ANOVA test followed by Dunnett's
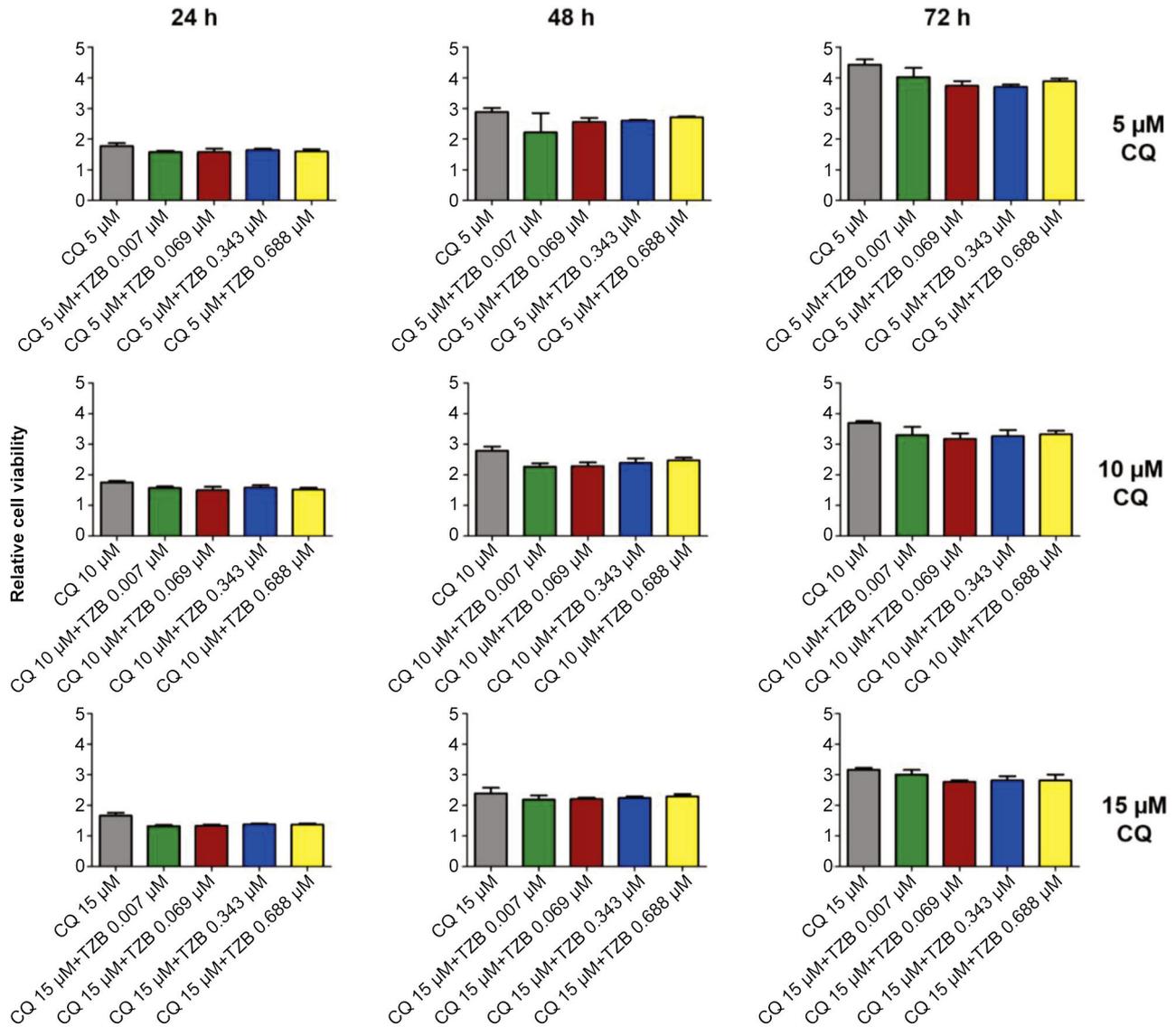
Figure 4. *Average relative cell viability±standard deviation (SD) for single agent chloroquine (CQ) and CQ+trastuzumab (TZM) concentrations at 24, 48 and 72 h (n=4; data represent average cell viability±SD).*

multiple comparison test. These analyses revealed that generally for the 24- and 72-h time points, the mean relative cell viability of single agent CQ at concentrations 5, 10 and 15 μM was significantly different from the mean relative cell viability of combinations containing these same concentrations of CQ. Overall, these results agree well with the synergistic interaction between CQ and TZM, as estimated *via* the interaction parameter Ψ. Additionally, the mean-transit-time parameter τ, which represents the time for each drug to achieve apoptosis of JIMT-1 cells, was estimated to be twice longer for CQ than for TZM (17.7 *vs*. 7 h), suggesting that CQ anti-tumoral activity is time-dependent.

Conclusion

In summary, this work demonstrated that as single agents CQ and TZM are inefficacious on JIMT-1 cells, however their combinatorial effects are synergistic. This finding may support the primary hypothesis of CQ lifting TZM resistance in HER2-positive BC refractory to TZM therapy. However, our findings also suggest that despite the fact that CQ and TZB act synergistically *in vitro* for the killing of JIMT-1 cells, the clinical utility of this combination may be limited due to CQ induced toxicity. Further *in vivo* studies could be considered to investigate whether CQ cytotoxic effects can

be achieved while keeping drug levels within therapeutically acceptable ranges.

Conflicts of Interest

The Authors declare that there are no conflicts of interest.

Authors' Contributions

YLF designed the study, conducted the experiments, and wrote the manuscript. CK helped conducting the experiments, SAO designed the study, provided scientific mentoring to all co-authors, and wrote and edited the manuscript.

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