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RESEARCH REPORT

Pharmacodynamics of cisplatin, methotrexate, and doxorubicin in an *in vitro* pharmacokinetics / pharmacodynamics model employing T24 human bladder cancer cells: the role of albumin under single administration-simulating conditions

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Abstract: *Background:* Bladder cancer, predominantly urothelial carcinoma, is a common malignancy. Despite the range of available treatments, recurrence and resistance remain significant challenges, thereby underscoring the need for the development of predictive *in vitro* pharmacokinetics / pharmacodynamics (PK/PD) models in order to optimize therapies. *Aim:* This study aimed at exploring the cytotoxic efficacy of three established anticancer agents (namely cisplatin, methotrexate, and doxorubicin) against T24 human bladder cancer cells, while using a novel *in vitro* PK/PD model. A key objective was to analyse the impact of 5% human albumin (simulating plasma protein binding) on each drug's cytotoxic efficacy, over a 72-h period. *Methodology:* A dynamic, peristaltic pump-based *in vitro* PK/PD system was employed, while cell growth inhibition was assessed spectrophotometrically using the reduction of the relative optical density at 630 nm as a surrogate for cell viability. Dose–response analyses were conducted for each drug at five different concentrations, in the absence and presence of 5% human albumin. Data were fitted to the E_{max} model and were validated with R^2 values. *Results:* All three of the herein assessed drugs (cisplatin, methotrexate, and doxorubicin) demonstrated a significant and concentration-dependent inhibition of T24 proliferation, with highest efficacies exhibited by cisplatin (followed by methotrexate then doxorubicin); all confirming a strong fit to the E_{max} model. The addition of albumin caused a plateauing effect in the obtained dose–response curves for all of the assessed chemotherapeutic agents, thereby indicating a reduced free drug availability due to protein binding, without completely abolishing the drug efficacy. *Conclusion:* This study established a robust *in vitro* PK/PD model for simulating drug effects on T24 cells, with the presence of albumin modulating the drug efficacy by limiting the free drug concentrations in the medium.

Keywords: albumin; cisplatin; bladder cancer; doxorubicin; methotrexate

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Bladder cancer is a prevalent malignancy, with urothelial carcinoma being its most common subtype. The incidence of bladder cancer is strongly influenced by risk factors such as smoking, occupational chemical exposure, and age, with a significant disparity between genders (Richters *et al.*, 2020; Saginala *et al.*, 2020). The disease is clinically categorized as non-muscle-invasive

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Table 1. Overview of the coefficient of determination (R^2) values generated by the study's experiments.

Chemotherapeutic agent tested	**Concentration** (mg/L)	R^2**-value in the absence of albumin**	R^2**-value in the presence of albumin**
cisplatin	150	0.8972	0.9523
	200	0.7960	0.9255
	250	0.9143	0.9611
	300	0.9293	0.9513
	350	0.9741	0.9699
methotrexate	40	0.9919	0.9613
	50	0.9814	0.9606
	60	0.9795	0.9545
	70	0.9760	0.9414
	80	0.9750	0.9112
doxorubicin	30	0.9659	0.9352
	40	0.9647	0.8957
	50	0.9587	0.9123
	60	0.9084	0.9541
	70	0.9108	0.9524
control	n/a	0.9692	0.9950

or muscle-invasive, with the first category comprising about 75% of initial diagnoses and carrying a high risk of recurrence, thereby necessitating rigorous monitoring and the adoption of improved treatment strategies (Witjes *et al.*, 2021; Babjuk *et al.*, 2022).

Standard treatments for bladder cancer include surgery, chemotherapy, and immunotherapy; however, cancer recurrence and resistance to treatment remain major challenges. This underscores the critical need for the development of predictive preclinical tools for the assessment of new therapies against this type of cancer. In this regard, *in vitro* pharmacokinetics / pharmacodynamics (PK/PD) models have been proven invaluable in simulating human drug responses, optimizing dosing, and designing rational combination therapies before clinical application (Saginala *et al.*, 2020). A crucial factor often overlooked in such models is the impact of plasma protein binding. Human serum albumin – the most abundant plasma protein – can bind to chemotherapeutic agents, thereby altering their pharmacodynamics by reducing the fraction of free (i.e., pharmacologically-active) drug available to reach tumour cells (Yu *et al.*, 2022).

This study aimed at assessing the pharmacodynamics of three established anticancer agents (namely cisplatin, methotrexate, and doxorubicin) in exerting cytotoxicity on T24 human bladder cancer cells, while using a novel *in vitro* PK/PD model. A key objective of the study was to analyse the impact of 5% human albumin (as a way of simulating the *in vivo* plasma protein binding) on each of the herein assessed drug's cytotoxic efficacy, over a 72-h period.

Methodology

In vitro PK/PD model setup

The herein employed experimental model has made use of a peristaltic pump-driven (MINIPULS Evolution; Gilson Inc., Villiers-le-Bel, France) *in vitro* PK/PD system, representing the first implementation of its kind in Iraq for assessing the pharmacodynamics of anticancer drugs. This dynamic model was specifically designed in order to reproduce clinically-relevant drug pharmacokinetics by maintaining time-dependent changes in drug concentrations rather than by relying on static exposures. After drugs are introduced into the system, their concentrations are gradually reduced in accordance with their reported $t_{1/2}$ values (ranging from 3 to 5 days), thereby reflecting realistic elimination kinetics and allowing for a more accurate simulation of the drug behavior in the human body.

Cell culturing of the T24 cells

The T24 human urinary bladder cancer cells (HTB-4™; obtained from ATCC) were cultured under standard conditions (37°C; 5% CO_2) in RPMI-1640 growth medium (Sigma-Aldrich, Germany), and were subsequently seeded into the PK/PD chambers with semipermeable cellulose membranes (Float-A-Lyzer; Spectrum Europe BV, Breda, Netherlands) at a 2×10^4-seeding density so as to assess the consistency of the drug activity across different conditions. Cells were allowed to adhere and establish growth prior to their exposure to the drugs.

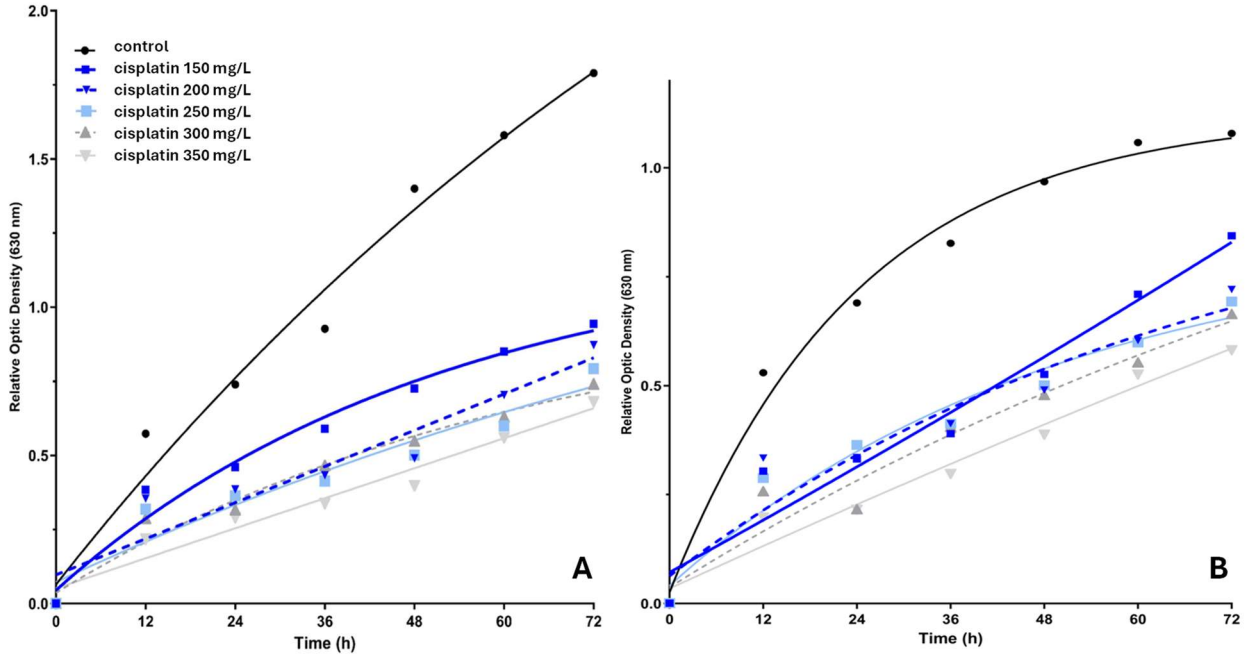

Figure 1. Cytotoxic effects of cisplatin (C_{max}: 150, 200, 250, 300, and 350 mg/L) on T24 cells, in the absence (A) and in the presence (B) of 5% human serum albumin in the culture medium.

Drug preparation and treatments

The three anticancer drugs were tested at five graded concentration levels: cisplatin (Accord, UK) was assessed at concentrations of 150–350 mg/L (Kehoe *et al.*, 1992), methotrexate (Koçak Farma, Turkey) was assessed at concentrations of 40–80 mg/L (Howard *et al.*, 2016), while doxorubicin (Koçak Farma, Turkey) was assessed at concentrations of 30–70 mg/L (Danhauser-Riedl *et al.*, 1993), thereby allowing for the generation of full dose–response curves for each agent. In order to investigate the effect of plasma protein binding on the drugs' pharmacodynamics, two treatment conditions were compared: one in the absence and one in the presence of 5% human serum albumin (Kedrion Biopharma, Italy) in the growth medium.

Measurement of cell viability

Cell viability and growth inhibition were monitored through the reduction of the relative optical density (ROD), which served as a surrogate marker of tumour cell proliferation. Measurements were performed using a spectrophotometer (BioTek, USA) at a wavelength of 630 nm, at multiple time intervals over a 72-h period.

Data analysis

The pharmacodynamic response for each drug was modelled using the E_{max} model; a widely accepted non-linear regression model for PK/PD studies. This has allowed for the estimation of the maximum achievable drug effect (E_{max}). Goodness-of-fit was assessed by calculating the coefficient of determination (R^2) values for each dose–response curve, thereby ensuring the reliability of the fitted models. Finally, comparisons between treatments in the absence and presence of albumin have provided insight into the magnitude of plasma protein binding-related effects on the pharmacological activity of the herein tested drugs, with p-values below 0.05. Data were analysed using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA, USA).

Results

Pharmacodynamics of cisplatin

The analysis of the cytotoxic effects of the herein assessed five different cisplatin concentrations (150, 200, 250, 300, and 350 mg/L) on T24 cells has revealed sigmoid dose–response curves with variable slopes in the absence (Figure 1A; $p<0.001$ for all cisplatin concentrations *versus* control) and in the presence (Figure 1B; $p<0.001$ for all cisplatin concentrations *versus* control) of 5% human serum albumin in the medium. The corresponding R^2 values are presented in Table 1.

Pharmacodynamics of methotrexate

The analysis of the cytotoxic effects of the herein assessed five different methotrexate concentrations (40, 50, 60, 70, and 80 mg/L) on T24 cells has revealed sigmoid dose–response curves with variable slopes in the absence (Figure 2A; $p<0.001$ for all methotrexate concentrations *versus* control) and in the presence (Figure

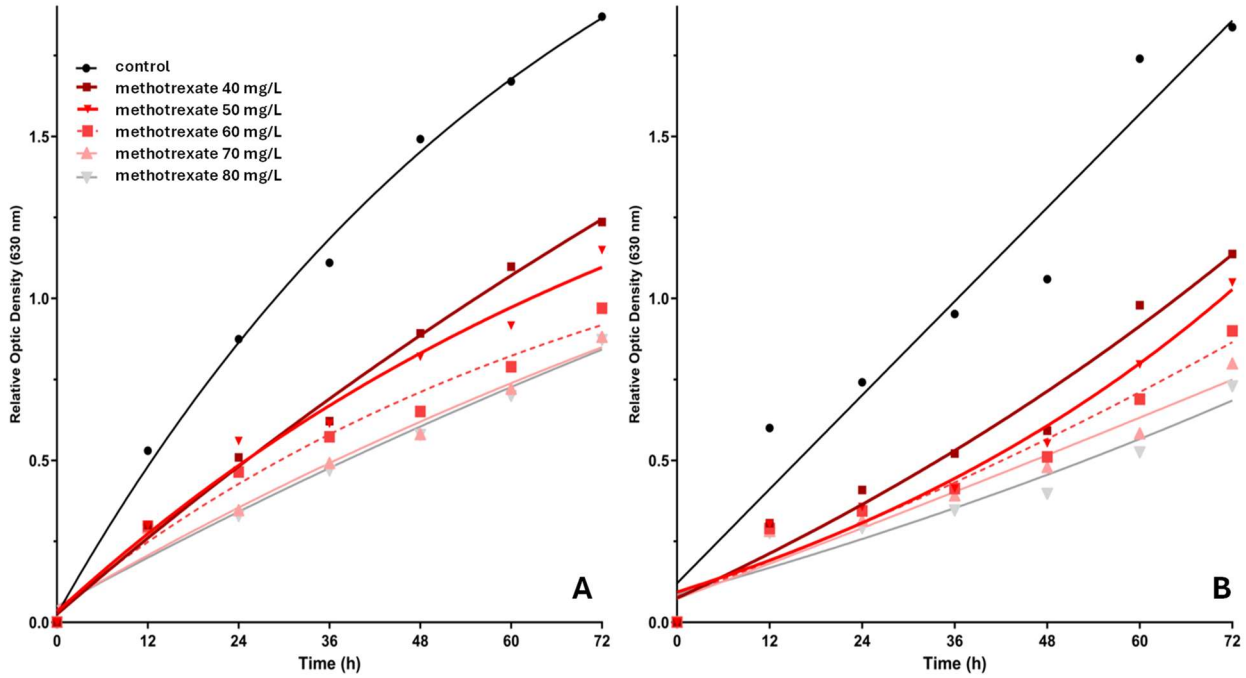

Figure 2. Cytotoxic effects of methotrexate (C_{max}: 40, 50, 60, 70, and 80 mg/L) on T24 cells, in the absence (A) and in the presence (B) of 5% human serum albumin in the culture medium.

2B; $p<0.001$ for all methotrexate concentrations *versus* control) of 5% human serum albumin in the medium. The corresponding R^2 values are presented in Table 1.

Pharmacodynamics of doxorubicin

The analysis of the cytotoxic effects of the herein assessed five different doxorubicin concentrations (30, 40, 50, 60, and 70 mg/L) on T24 cells has revealed sigmoid dose–response curves with variable slopes in the absence (Figure 3A; $p<0.001$ for all doxorubicin concentrations *versus* control) and in the presence (Figure 3B; $p<0.001$ for all doxorubicin concentrations *versus* control) of 5% human serum albumin in the medium. The corresponding R^2 values are presented in Table 1.

Discussion

The current study has employed an *in vitro* PK/PD system in an attempt to simulate the inhibitory effects of three established chemotherapeutic agents (namely cisplatin, methotrexate, and doxorubicin) on the proliferation of human bladder cancer T24 cells. To the best of our knowledge, this marks the first implementation of an *in vitro* PK/PD model for the assessment of the pharmacodynamics of anticancer agents in Iraq. By employing this advanced simulation technique, a more realistic and controlled environment was established, contributing to an improved understanding of the pharmacological profiles and the interactions of the tested drugs under clinically-relevant *in vitro* conditions.

This model has enabled the dynamic monitoring of drug effects over a 72-h period, using ROD as a surrogate for cell growth and viability. The use of ROD as a growth index is well established and correlates closely with viable cell numbers in culture *via* the crystal violet procedure, particularly when monitored over time in a controlled incubation system (Iveson *et al.*, 2018). The sigmoid growth curves obtained have validated the robustness of ROD as a quantitative endpoint for *in vitro* PK/PD analysis, as reflected in high R^2 values (Table 1), thereby indicating a strong fit of the data to the E_{max} model. These findings reinforce the suitability of spectrophotometric readouts for characterizing anticancer drug effects over time in dynamic *in vitro* systems (Chunarkar-Patil *et al.*, 2024).

The pharmacodynamic profile of cisplatin, across concentrations ranging from 150 to 350 mg/L, demonstrated a typical sigmoidal inhibition pattern consistent with concentration-dependent cytotoxicity. Each tested concentration exhibited significantly greater inhibitory effects than the untreated control, thereby indicating a strong dose–response relationship within the model (Boulikas and Vougiouka, 2003). Cisplatin's mechanism of action involves DNA crosslinking, leading to apoptosis through the disruption of the replication and transcription processes. Its concentration-dependent cytotoxicity pattern has been previously established across various cancer cell types, including urothelial carcinoma (Abe *et al.*, 2020; Bahremand *et al.*, 2024). Importantly, the clear dose-responsive inhibition observed in this study affirms that the PK/PD system accurately replicates *in vitro* the pharmacological effects of cisplatin.

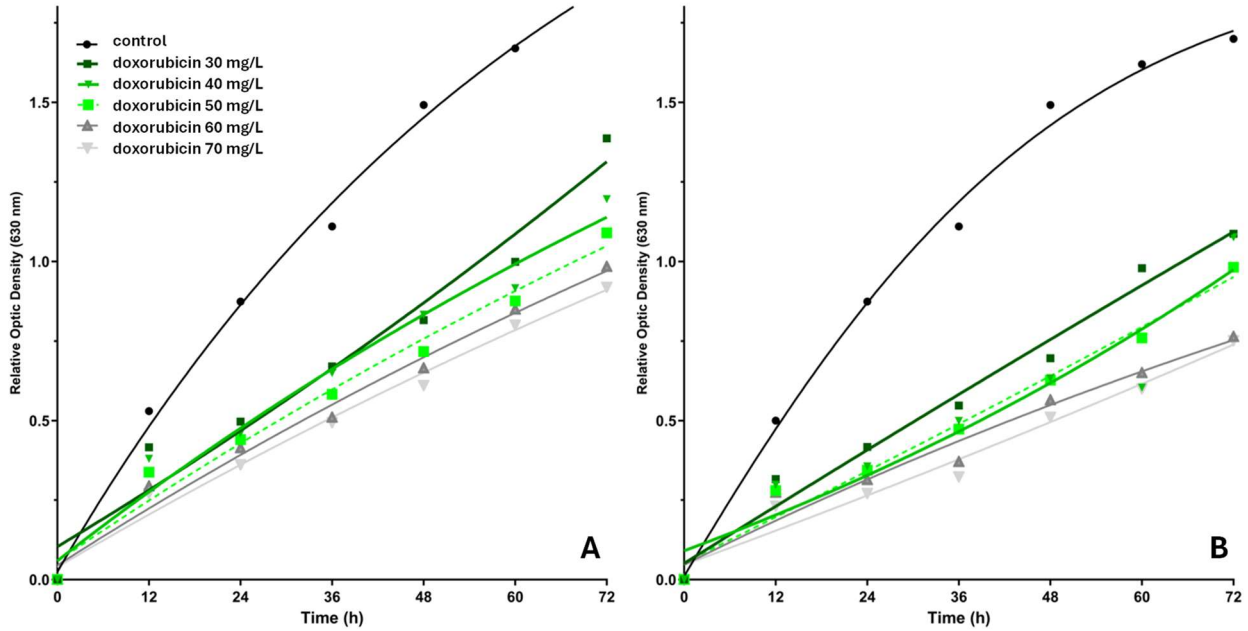

Figure 3. Cytotoxic effects of doxorubicin (C_{max}: 30, 40, 50, 60, and 70 mg/L) on T24 cells, in the absence (A) and in the presence (B) of 5% human serum albumin in the culture medium.

Both cisplatin and doxorubicin are known to be sensitive to light exposure and prone to adsorption onto surfaces, which can significantly affect their stability and concentration during the undertaking of *in vitro* experiments (Greco *et al.*, 2023). In order to mitigate these effects, the entire *in vitro* PK/PD system was carefully shielded from light by wrapping it in aluminum foil, thereby preventing the photodegradation of these drugs. Furthermore, efforts were made to minimize the contact with plastic materials, which are known to facilitate drug binding. As a result, the setup primarily utilized glass beakers and cellulose-based dialysis membranes, with plastic tubing as the only necessary non-glass component present. These precautions were implemented so as to ensure the integrity and accuracy of the drug exposure throughout the duration of the experiment (Abdalwahd *et al.*, 2024). Moreover, the employed peristaltic pump-based *in vitro* PK/PD model offers flexibility for further adaptation. For instance, physiological factors such as serum albumin can be incorporated into the system in order to better simulate *in vivo* conditions, allowing for the investigation of drug–protein interactions and their influence on pharmacodynamics and microbial or tumour cell responses (Jihad *et al.*, 2024).

The addition of 5% human albumin to the cisplatin treatments has preserved the general inhibitory trend, with all tested concentrations still exhibiting significant growth suppression compared to controls (Figure 1B). Interestingly, while the R^2 values remained high (Table 1), the degree of additional inhibition plateaued across doses, with no significant differences being detectable among them. This suggests that albumin binding may slightly alter cisplatin's bioavailability or its free active fraction. Albumin is known to bind many chemotherapeutic drugs, potentially modulating their distribution and cellular uptake, and may therefore affect their pharmacodynamic effects *in vitro* and *in vivo* (Kratz, 2008). Yet, in the present model, albumin did not reverse or attenuate cisplatin's cytotoxic effects, indicating that its impact on cisplatin's efficacy may be limited in the bladder cancer microenvironment. The binding did not significantly reduce efficacy, suggesting that the free active fraction of cisplatin may still be sufficient for the induction of therapeutic activity within the T24 cellular microenvironment.

Albumin's addition to the medium has also led to a convergence of the inhibitory effects of methotrexate across the assessed concentrations (Figure 2B), thereby reflecting a likely saturation phenomenon that limits the variability of the response to the chemotherapeutic agent (Khalaila *et al.*, 2006). This did not significantly alter the overall pattern of inhibition, although the degree of inhibition across different concentrations appeared to converge. This may indicate a saturation effect, where albumin binding reduces free drug availability equally across the concentrations of methotrexate, resulting in a plateau in efficacy (Widemann and Adamson, 2006). Similar trends have been noted in pharmacokinetic studies, in which the methotrexate–albumin binding leads to reduced renal clearance, and also limits the agent's intracellular uptake. Thus, albumin may serve as a modulator of methotrexate pharmacokinetics without dramatically changing its cytotoxic (pharmacodynamic) profile under static *in vitro* conditions (Tang *et al.*, 2024).

Finally, while the addition of human albumin to doxorubicin resulted in the smallest observable effect on the T24 cells' viability among the drugs tested, this plateauing remains a significant finding. It suggests that although a substantial portion of doxorubicin binds to albumin (thereby reducing the free fraction available for cellular uptake), the unbound drug remains highly potent. This is consistent with the known high affinity of doxorubicin for human serum albumin, which can modulate its distribution, efficacy, and toxicity profiles *in vivo* (Kratz, 2008; Zhao *et al.*, 2019).

Conclusion

This study has established an *in vitro* PK/PD model for simulating drug effects on T24 cells, with the presence of albumin modulating the drug efficacy by limiting the free drug concentrations in the medium. The study has assessed the impact of human albumin on the pharmacodynamics of cisplatin, methotrexate, and doxorubicin, concluding that the presence of albumin in the culture medium can cause a plateauing effect in the obtained dose–response curves for all of the assessed chemotherapeutic agents, without completely abolishing their cytotoxic efficacy. The herein used *in vitro* PK/PD model could prove a valuable tool for the preclinical assessment of drugs under clinically-relevant, single administration-simulating conditions.

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Conflicts of interest statement

None to declare.

Data availability statement

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

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