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Research Article

Comprehensive Statistical Analysis of the Pharmacokinetics, Safety and Clinical Benefit Rate of Mitotam in a Single-Center Phase I/Ib Trial in Patients with Metastatic Solid Tumors

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Abstract

Background: Mitochondrially Targeted Tamoxifen (MitoTam), the first mitochondrial inhibitor to disrupt Complex I (CI)-dependent respiration, previously showed antitumor activity against Renal Cell Carcinoma (RCC) with a good safety profile. We investigated the relationships of Pharmacokinetic (PK) parameters, biodistribution and patient baseline diagnosis with the clinical outcome and toxicity of Mitotam.

Methods: In the phase I/Ib MitoTam-01 trial, patients with metastatic solid tumors were treated with Mitotam monotherapy. PK parameters were calculated separately for the doses used in both trial phases. Data were analyzed descriptive analyses and using the generalized linear model framework as stochastic test.

Results: The non-compartmental analysis of PK parameters showed that the extent of exposure was positively correlated with the dose. Most of the PK profiles suggested that MitoTam was redistributed from the tissues or from protein binding back into the blood circulation, with very low accumulation. The exposure efficacy relationship did not show significant differences between responders and non-responders in phase Ib. However, the Area Under the Curve from time zero to time (AUC0-t) and Maximum Concentration (Cmax) values were greater in Renal Cell Carcinoma (RCC) patients than in responders with other diagnoses. These data are consistent with the preclinical findings showing preferential MitoTam accumulation in kidneys and the high clinical benefit rate in RCC patients in the phase Ib part.

Conclusion: These comprehensive analyses demonstrate the impact of MitoTam on the clinical benefit rate that is related to the dose and corresponding PK parameters, as well the underlying diagnosis. The PK data supported the previously recommended dose of 3.0 mg/kg weekly for future trials.

Keywords: Mitotam; Pharmacokinetics; Phase I/Ib; Clinical benefit rate; Safety; Renal cell carcinoma

Introduction

Many mitochondrial pathways, including Oxidative Phosphorylation (OXPHOS), fatty acid, glutamine and one-carbon metabolism, are altered in tumors due to mutations in oncogenes, tumor suppressor genes and metabolic enzymes [1]. Mitocans are anticancer agents that act *via* targets on or within mitochondria. However, their translation from preclinical experiments has been challenging and only a few compounds have entered early-stage clinical trials [1]. To date, only one mitochondria-targeting agent (venetoclax) has been approved for Chronic Lymphocytic Leukemia (CLL) and Acute Myeloid Leukemia (AML) [2]. Negative outcomes of several clinical trials [1,3-5] were critically assessed and generalized to the entire research concerning mitochondrial targeting [6]. Our research shows that targeting mitochondria is a plausible anticancer therapeutic strategy.

Mitochondrially Targeted Tamoxifen (MitoTam) is a Triphenylphosphonium (TPP+) tagged mitocan that interferes with mitochondrial functions and Complex I (CI)-dependent respiration [7]. By 'intercalating' into the inner mitochondrial membrane, MitoTam dissipates the mitochondrial membrane potential, promoting both apoptosis and necroptosis [8]. The anticancer effect of MitoTam against Renal Cell Carcinoma (RCC) was as efficient as immunotherapy with immune checkpoint inhibitors in an animal model [8].

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The clinical efficacy and safety of MitoTam were evaluated in the phase I/Ib MitoTam-01 trial.9 we reported a Clinical Benefit Rate (CBR) of MitoTam of 37% (14/38) in patients with metastatic solid tumors, regardless of the diagnosis, and of 83% (5/6) among patients with RCC enrolled in the phase Ib part. All patients except one with CBR achieved disease stabilization following Mitotam treatment, as measured by RECIST 1.1 criteria. The maximum tolerated dose was 5.0 mg/kg, the penultimate dose in the series of nine doses tested. Systemic toxicities were mainly hematological Adverse Events (AEs) and fever. Local toxicities were related to the administration route, including risk of Thromboembolic (TE) complications.

The Pharmacokinetic (PK) analysis was a primary endpoint of the MitoTam-01 trial to determine the total exposure, optimal dosing frequency and potential accumulation of MitoTam. The initial routine PK analysis [9], performed by an external evaluator, led us to more detailed and accurate assessment of results. In this article, we report a comprehensive and amended exploration of Mitotam PK, including the identification of parameters that may improve the odds of a clinical benefit or influence its toxicity. Herein, we also evaluated marginal PK parameters to obtain more insightful results. To obtain more robust data on the safety and efficacy of MitoTam, the original elimination half-life [9], was re-evaluated in this publication as distribution serum half-life (T½α) and terminal serum elimination half-life (T½β). We also report correlations between the CBR and PK parameters, including a critical assessment of their reliability, focusing on treatment regimens and number of treatment cycles, dose, sex, baseline diagnosis and histogenetic origin of the disease.

Materials and Methods

Study design

MitoTam-01 was an open-label, single-arm, non-randomized, single center phase I/Ib trial that evaluated the safety and efficacy of Mitotam. The study design was previously reported (EudraCT 2017-004441-25; registration date: 01st November, 2017) [9]. All patients had previously undergone systemic anticancer therapy (three in median) that had been terminated. The ClinPK reporting checklist was consulted in the preparation of this report [10].

The phase I study evaluated the safety of a single cycle of Mitotam across nine escalating doses and two treatment schemas. Phase Ib evaluated the efficacy and safety of repeated doses of Mitotam in three different regimens. Supplemental Tables S1-S7 present further information about the study design, including dosing and treatment schemes, administration of Mitotam, prohibited medications and timing of blood sampling.

Mitotam analysis

The analysis of Mitotam and the Internal Standard (IS) MitoTam-D15 are described in detail in the supplemental methods.

Determination of PK parameters

Phoenix WinNonlin® software version 8.1 (Certara, USA) was used to calculate the PK parameters. Non-compartmental modeling used the linear trapezoidal/linear interpolation calculation methods. The best-fit method with uniform weighting was used to calculate the terminal elimination rate constant distribution and elimination half-life. Serum concentrations below the lower limit of quantification were set to zero. The following PK parameters were estimated for each subject, sampling day, and cycle: Maximum serum Concentration (Cmax), Area Under the serum concentration curve (AUC0-t), time to reach the maximum serum concentration (Tmax), distribution serum half-life (T½α), terminal serum elimination half-life (T½β), Mean Residence Time (MRT), Serum Clearance (CL), Volume of distribution (Vz) and accumulation index. Analysis of PK variance was used to test the effects of phase, cohort, cycle, day and sex at a 5% significance level for AUC0-t and Cmax. PK parameters requiring extrapolation of the elimination phase to infinity (T½α, T½β, MRT, and CL) were not considered as the main parameters in statistical correlation analysis. These extrapolated PK parameters should be evaluated cautiously.

Statistical analysis

Descriptive statistics, including the empirical mean, Standard Deviation (SD), empirical median, minimum, first quartile (i.e. 25th percentile), empirical median, third quartile (i.e. 75th percentile) and maximum, were calculated for the key variables. These parameters are also presented as matrices in pairwise plots. Where appropriate we used random effects mixed models (Generalized Linear Mixed Models (GLMM)) in addition to the descriptive PK analysis.

The theoretical test level was set to 0.05. Results with p values of <0.05 were considered statistically significant and reported as such in this manuscript. R statistical software by R Core Team (2021) version 4.1.1 (released on 10th August, 2021) was used [11]. All of the stochastic model formulations together with the estimates of the statistically significant model parameters and corresponding p-values are given in Appendix A.

Results

Patient characteristics

Seventy-five patients were enrolled between 23rd May, 2018, and 22nd July, 2020, comprising 37 in phase I and 38 in phase Ib. Their characteristics are summarized in Table 1 and Supplemental Tables S8 and S9. We found no significant differences in the baseline characteristics among treatment groups that could be relevant to the exposure, distribution, metabolism, or clearance of MitoTam that might influence its PK.

Pharmacokinetics

Serial PK data were analyzed for three-times-weekly (n=27) and once-weekly (n=10) dosing schemes in phase I and in phase Ib (n=20 *vs.* n=18). Table 2 shows The PK parameters for the nine studies in phase I (0.25-6.0 mg/kg) and three in phase Ib (1.0, 3.0 and 4.0 mg/kg).

The results suggested a two-compartment model. However, we observed large variability in the serum concentrations and PK parameter estimates among the patients in all included studies in both phases. The serum concentration time profiles and derived non-compartmental estimated systemic CL and Vz for all studies in both phases did not reach the hepatic blood flow and largely exceeded the total body water in humans (1450 mL/min and 42000 mL, respectively, in a human with body weight of 70 kg; approximately 1243 mL/h/kg and 600 mL/kg), suggesting a low extraction ratio and large distribution into tissues. Most of the PK profiles and serum levels indicated possible redistribution of MitoTam from the tissues back into the serum. During the release of MitoTam from tissue into the blood, its concentrations were often higher than at the time immediately after the end of intravenous administration. The redistribution of MitoTam together with the small numbers of patients in individual groups likely led to the relatively large SD for Tmax. The PK results of the individual studies in phases I and Ib are summarized below and in Tables 2 and 3.

	Dose of MitoTam, mg/kg (n)	Age, years, mean (SD)	Sex (M/F)	Height, cm, mean (SD)	Weight, kg, mean (SD)	BMI, kg/m2, mean (SD)
Phase I	0.25 (4)	59.75 (9.36)	02-Feb	172.75 (9.91)	72.67 (16.05)	24.61 (2.59)
	0.5 (6)	50.67 (8.84)	02-Apr	167.00 (7.66)	69.83 (19.19)	24.85 (5.87)
	1.0 (3)	47.33 (3.30)	02-Jan	166.67 (13.82)	83.67 (4.19)	30.55 (3.96)
	1.5 (3)	59.67 (5.44)	02-Jan	172.00 (10.71)	106.33 (13.57)	36.66 (8.35)
	2.25 (6)	62.17 (8.32)	02-Apr	167.00 (8.91)	78.20 (13.78)	28.03 (3.93)
	3.0 (5)	61.40 (6.80)	02-Mar	169.80 (11.57)	83.60 (17.00)	29.22 (6.74)
	4.0 (3)	65.33 (8.06)	01-Feb	165.33 (5.91)	66.33 (6.65)	24.46 (3.78)
	5.0 (6)	66.50 (3.59)	04-Feb	172.67 (8.73)	83.83 (17.18)	27.79 (3.72)
	6.0 (1)	68.00 (0.00)	1/0	180.00 (0.00)	87.00 (0.00)	26.85 (0.00)
Phase Ib	1.0 (20)	59.05 (9.66)	12-Aug	172.35 (9.71)	77.16 (18.37)	26.25 (5.10)
	3.0 (9)	63.78 (8.69)	05-Apr	167.67 (8.62)	79.11 (17.85)	28.06 (5.77)
	4.0 (9)	63.56 (5.54)	06-Mar	176.22 (8.40)	83.89 (13.15)	27.01 (3.95)

Note: SD: Standard Deviation; M: Males; F: Females; BMI: Body Mass Index

Table 1: Demographic data of enrolled patients (N=75).

	Phase I, study 0.25 mg/kg (n=4)					
	Mean	**SD**	**Median**	**Q1**	**Q3**	**IQR**
AUC0-t (ng*h/mL)	960.81	740.59	499.2	280	1720	1440
Cmax (ng/mL)	302.7	223.46	304.5	133.5	385	251.5
Tmax (h)	0.43	0.43	0.5	0.08	0.5	0.42
T½α (h)	1.41	2.07	0.55	0.4	1.45	1.05
CL (mL/h/kg)	412.08	314.56	299.55	136.43	725.37	588.95
Vz (mL/kg)	7315.35	7843.77	3527.9	2919.73	11661.85	8742.13
Accumulation index	1.16	0.22	1.08	1	1.29	0.29
Phase I, study 0.5 mg/kg (n=6)						
AUC0-t (ng*h/mL)	4447.31	7805.87	1495.4	979.1	2148.9	1169.8
Cmax (ng/mL)	1037.8	1523.36	500	229	764	535
Tmax (h)	0.11	0.1	0.083	0.083	0.083	0
T½α (h)	1.26	0.72	0.9	0.7	2	1.3
CL (mL/h/kg)	265.91	167.58	271.1	127.7	404.65	276.95
Vz (mL/kg)	11023.61	8281.26	10019.00	6713.15	13545.78	6832.63
Accumulation index	1.51	0.34	1.43	1.2	1.75	0.55
Phase I, study 1.0 mg/kg (n=3)						
AUC0-t (ng*h/mL)	3068.52	1535.63	2160.3	1925.15	4405.6	2480.45
Cmax (ng/mL)	1019.11	772.26	669	469.5	1565	1095.5
Tmax (h)	0.27	0.21	0.08	0.08	0.5	0.42
T½α (h)	0.81	0.43	0.6	0.4	1.3	0.9
CL (mL/h/kg)	342.28	123.22	403.5	186.1	447.58	261.48

Vz (mL/kg)	9588.68	5745.49	8293.8	4887.75	14132.03	9244.28
Accumulation index	1.21	0.16	1.21	1.07	1.3	0.22
Phase I, study 1.5 mg/kg (n=3)						
AUC0-t (ng*h/mL)	14595.87	24 224.16	3925	3115.8	15371.30	12255.50
Cmax (ng/mL)	2486.55	2424.98	1530	723	4180	3457
Tmax (h)	1.7	3.72	0.08	0.08	1.75	1.67
T½α (h)	1.32	1.98	0.34	0.24	1.62	1.37
CL (mL/h/kg)	379.08	221	356	177.95	591.75	413.8
Vz (mL/kg)	9975.18	6374.44	10149.30	3601.5	16261.80	12660.30
Accumulation index	1.17	0.1	1.2	1.06	1.27	0.21
Phase I, study 2.25 mg/kg (n=6)						
AUC0-t (ng*h/mL)	22250	17 553.87	13461.75	8242	37726.73	29484.73
Cmax (ng/mL)	2734.68	1509.56	2435	1547.5	3450	1902.5
Tmax (h)	1.74	3.25	0.5	0.08	0.5	0.42
T½α (h)	1.17	0.77	1.04	0.68	1.65	0.97
CL (mL/h/kg)	171.81	160.47	111.2	51.55	316.2	264.65
Vz (mL/kg)	5347.69	6527.47	2174.9	1357.55	8769.35	7411.8
Accumulation index	1.22	0.16	1.19	1.08	1.35	0.27
Phase I, study 3.0 mg/kg (n=5)						
AUC0-t (ng*h/mL)	23783.81	17 643.06	14704.70	12005.55	39495.65	27490.10
Cmax (ng/mL)	4204	3739.03	3040	1867.5	5035	3167.5
Tmax (h)	0.33	0.12	0.25	0.25	0.5	0.25
T½α (h)	1.89	1.45	1.38	0.94	2.5	1.56
CL (mL/h/kg)	177.22	109.53	225.4	50.5	279.85	229.35
Vz (mL/kg)	4384.36	2887.41	5406.8	1000.05	7257.45	6257.4
Accumulation index	1.14	0.08	1.15	1.06	1.2	0.14
Phase I, study 4.0 mg/kg (n=3)						
AUC0-t (ng*h/mL)	27421.30	2487.66	28932.00	23914.40	29417.50	5503.1
Cmax (ng/mL)	3311.67	824.04	2980	2510	4445	1935
Tmax (h)	0.33	0.12	0.25	0.25	0.5	0.25
T½α (h)	1.86	0.32	1.93	1.45	2.22	0.77
CL (mL/h/kg)	141.43	13.55	132.1	131.6	160.6	29
Vz (mL/kg)	9135.16	1612.07	8434.6	7606.6	11364.30	3757.7
Accumulation index	x	x	x	x	x	x
Phase I, study 5.0 mg/kg (n=6)						
AUC 0-t (ng*h/mL)	47833.12	36 209.78	36341.20	21513.58	66428.85	44915.28
Cmax (ng/mL)	6395	3772.63	6100	2670	10067.50	7397.5
Tmax (h)	1.25	2.13	0.25	0.25	1.88	1.63
T½α (h)	10.82	17.37	1.7	0.33	21.53	21.2
CL (mL/h/kg)	142.52	65.56	133.15	87.3	216.6	129.3

Vz (mL/kg)	9858.4	6127.42	7716.75	4221.05	17917.28	13696.23
Accumulation index	x	x	x	x	x	x
Phase I, study 6.0 mg/kg (n=1)						
AUC0-t (ng*h/mL)	119 682.50	0	119 682.50	59841.25	59841.25	0
Cmax (ng/mL)	15 800	0	15 800	7900	7900	0
Tmax (h)	0.25	0	0.25	0.125	0.125	0
T½α (h)	5.39	0	5.39	2.7	2.7	0
CL (mL/h/kg)	49.7	0	49.7	24.85	24.85	0
Vz (mL/kg)	1964.3	0	1964.3	982.15	982.15	0
Accumulation index	x	x	x	x	x	x

Note: SD: Standard Deviation; Q1: First quartile; Q3: Third quartile; IQR: Interquartile Range; AUC0-t: Area Under the Curve; Cmax: Maximum (peak) serum concentration; Tmax: Time to maximum (peak) serum concentration; T½α: Distribution serum half-time; CL: Serum clearance; Vz: Distribution volume and x: Not done/uknown.

Table 2: Pharmacokinetic parameters of MitoTam for all studies and regimens in phases I.

	Phase Ib, regimen 1: 1.0 mg/kg (n=20)					
	Mean	**SD**	**Median**	**Q1**	**Q3**	**IQR**
AUC0-t (ng*h/mL)	34891.56	30941.63	28427.9	9929.95	49214.08	39284.13
Cmax (ng/mL)	4461.43	3497.12	3670	2116.25	6300	4183.75
Tmax (h)	0.42	0.34	0.25	0.25	0.5	0.25
T½α (h)	1.56	6.04	0.4	0.31	0.63	0.32
CL (mL/h/kg)	69.37	105.89	21.55	12.35	62.23	49.88
Vz (mL/kg)	2325.08	2467.06	1319.75	892.95	2813.28	1920.33
Accumulation index	2.34	3.37	1.61	1.14	2.15	1.01
Phase Ib, regimen 2: 3.0 mg/kg (n=9)						
AUC0-t (ng*h/mL)	38181.86	28141.25	29585.5	15334.95	53474.25	38139.3
Cmax (ng/mL)	5483	2991.03	4800	2860	7302.5	4442.5
Tmax (h)	0.67	1.64	0.5	0.25	0.5	0.25
T½α (h)	1.28	2.43	0.58	0.37	0.92	0.56
CL (mL/h/kg)	100.7	81.67	71	32.9	146.3	113.4
Vz (mL/kg)	1904.29	1090.85	1486.95	961.35	2711.57	1750.23
Accumulation index	1.01	0.02	1	1	1.01	0.01
Phase Ib, regimen 3: 4.0 mg/kg (n=9)						
AUC0-t (ng*h/mL)	39895.81	39727.58	28570	19430.3	44191.3	24761
Cmax (ng/mL)	6201.54	2722.02	6060	3980	7730	3750
Tmax (h)	1.01	3.73	0.5	0.25	0.5	0.25
T½α (h)	1.8	2.11	1.05	0.56	2.01	1.45
CL (mL/h/kg)	120.24	72.06	100.7	62.75	183.53	120.78
Vz (mL/kg)	2450.61	3498.82	1537.6	1129.48	2138.53	1009.05
Accumulation index	1.01	0.02	1	1	1.003	0

Note: SD: Standard Deviation; Q1: First quartile; Q3: Third quartile; IQR: Interquartile Range; AUC0-t: Area Under the Curve; Cmax: Maximum (peak) serum concentration; Tmax: Time to maximum (peak) serum concentration; T½α: Distribution serum half-time; CL: Serum clearance; Vz: Distribution volume and x: Not done/uknown.

Table 3: Pharmacokinetic parameters of MitoTam for all studies and regimens in phases Ib.

Phase I, doses 0.25-3.0 mg/kg

After intravenous administration on D1, D3 and D5, the Cmax was reached at the mean time of 0.82 h (SD 2.24) and the mean T½α was 1.24 h (SD 1.16) combining data from all these studies. Serum Mitotam levels were generally measurable 36 h post-dose.

The PK profiles of all subjects included in this phase declined in a multi-exponential manner. T½β ranged from 15.91 (SD 4.13) to 16.72 h (SD 7.73) for the 0.50-3.0 mg/kg studies and was 7.33 h (SD 4.69) for the 0.25 mg/kg study. The residual area was <20% in all subjects included in this study phase.

MRT and the total elimination time ranged from 7.19 to 15.73 h and 17.70 to 86.82 h, respectively, among the studies included in phase I. The mean estimated systemic CL of all phase I studies combined was 280.81 mL/h/kg (SD 214.41). The mean apparent Vz was 7679 mL/kg (SD 6915). The PK profiles and serum levels suggest that Mitotam may be rereleased from the tissues back into the serum. The mean accumulation index for the included cohorts was 1.27 (SD 0.26).

Phase I, doses 4.0-6.0 mg/kg

After a single intravenous dose, the Cmax was reached at the mean time of 0.88 h (SD 1.80). Mitotam was still measurable in serum at 168 h post-dose. The mean intensity and extent of exposure almost doubled between the 4.0 and 5.0 mg/kg studies, with Cmax of 3312 ng/mL (SD 824) *vs.* 6395 ng/mL (SD 3772) and AUC0-t of 27421 ng*h/mL (SD 2488) *vs.* 47833 ng*h/mL (SD 36210).

The 6.0 mg/kg cohort only included one subject, which prevented meaningful evaluation. The PK profiles of all subjects included in this study phase declined in a multi-exponential manner. T½β was 44.46 h (SD 4.49) and 46.28 h (SD 12.28) for the 4.0 and 5.0 mg/kg studies and 27.41 h in the single subject in the 6.0 mg/kg cohort.

The residual areas were <20% in all subjects included in this study phase. MRT and the total elimination time ranged from 25.04 to 34.28 h and 202.06 to 225.16 h, respectively, across the studies included in phase I. The mean estimated CL for all patients included in these cohorts was 132.91 mL/h/kg (SD 61.50). The mean Vz was 8852 mL/kg (SD 5645). The PK profiles and serum levels suggest that Mitotam may be rereleased from the tissue back into serum.

Phase Ib, doses 1.0-4.0 mg/kg

Mitotam was still measurable in serum at 24 h post-dose. There was no apparent relationship between the intensity or extent of exposure and increasing cycle number. The PK profiles of all subjects included in this study phase declined in a multi-exponential manner. The residual area was >20% for most of the subjects included in this study phase; therefore, the extrapolated PK parameters were not reliable in these cases. The T½β, when estimable and reliable, ranged from 6.19 to 12.46 h for the 1.0 mg/kg dose, from 6.73 to 9.49 h for the 3.0 mg/kg dose and from 6.17 to 11.62 h for the 4.0 mg/kg dose, considering all cycles. The MRT and time of total elimination, when estimable and reliable, ranged from 2.89 to 14.80 h and 30.82 to 56.91 h for the 1.0 mg/kg dose, from 7.43 to 10.74 h and 49.73 to 67.53 h for the 3.0 mg/kg dose, and from 7.66 to 12.21 h and 48.81 to 72.29 h for the 4.0 mg/kg dose, considering all cycles. T½α was significantly (p=0.007) longer for the 4.0 mg/kg dose than the 3.0 mg/kg dose. The PK profiles and serum levels suggest that Mitotam may be rereleased from tissue back into serum.

Because of the large inter-subject variability in the serum MitoTam concentrations and PK parameter estimates across all studies in both phases, we performed a longitudinal data analysis of T½β using GLMM in addition to the descriptive PK analysis for repeated observations across subjects. The PK analysis affirmed the significant effect of the dose on the AUC0-t and Cmax. The stochastic model formulations and the estimates of the statistically significant model parameters with corresponding p-values are given in Appendix A.

Exposure-efficacy relationship

We previously reported that the CBR was 37% (14/38) in the phase Ib part of the trial.9 The CBR was 30% in regimen 1, 78% in regimen 2 and 11% in regimen 3. Because the unexpected difference in the CBR between the weekly regimens 2 and 3 is unlikely to be explained by a difference in dose (3.0 *vs.* 4.0 mg/kg), we divided the patients into subgroups according to the histogenetic origin of the tumor (Supplementary Table S9). A significant CBR (p=0.018) was observed in tumors of Mesodermal (ME) origin with RCC being the most frequent diagnosis in this subgroup. In patients with RCC (n=6) treated in regimens 1 and 2, the CBR reached 83%. In this section, we summarize the relationship between PK parameters and the efficacy of MitoTam.

The statistical analysis using Generalized Linear Model (GLM) revealed non-significant effects of the PK parameters AUC0-t (borderline p=0.072) and Cmax (p=0.999) on the CBR (Appendix A). The PK parameters of responders and non-responders were also not significantly different (Table 4). In regimen 1, PK data from six responders and 14 non-responders were compared. In regimen 2, PK data from seven responders and two non-responders were evaluated. For regimen 3, there was one responder and eight non-responders. The mean AUC0-t was greater in responders than in non-responders in regimen 2 (42373.73 ng*h/mL (SD 29422.60) *vs.* 21223.37 ng*h/mL (SD 11552.67), respectively). The mean Cmax of responders was also greater than that of non-responders in regimen 2 (5908.97 ng/mL (SD 2980.33) *vs.* 3518.89 ng/mL (SD 672.14), respectively). The data in regimen 2 suggest a possible association between PK and CBR.

To better explain the CBR in patients with RCC, we compared the PK parameters between responders with RCC (n=5) and responders with other solid tumors (n=8) (Table 5). Patients with RCC had a greater exposure to MitoTam, and the AUC0-t and Cmax were greater in regimens 1 and 2. However, we did not confirm the hypothesis that the PK parameters of patients with RCC differ to those of patients with other solid tumors. A significantly greater number of responders in regimen 2 (including 3 patients with RCC) than in regimen 3 excluding patients with RCC (78% *vs.* 11%, p=0.001) supports the hypothesis that the CBR in regimen 2 is related to the diagnosis (i.e. RCC).

The responders could repeat MitoTam therapy according to the trial protocol. Therefore, it was interesting to observe the exposure to MitoTam over time in these patients. In regimen 1, four patients repeated treatment; one patient received eight cycles (16 weeks), two received 12 cycles (24 weeks) and one received 16 cycles (32 weeks). Similarly, in regimen 2, one patient with a clinical benefit received 10 cycles (10 weeks) and two patients received 12 cycles (12 weeks) (Supplemental Table S9). MitoTam exposure did not change with increasing number of cycles, regardless of the treatment scheme (three times a week, biweekly *vs.* once weekly) and regimen (dose 1.0 *vs.* 3.0 mg/kg). An increase in MitoTam accumulation was not observed these cases. Grade 3 (G3) systemic AEs were not observed in the subset of responders with prolonged MitoTam treatment. Thus, repeating treatment cycles can be considered safe with a low risk of AEs.

	Regimen 1: Responders (n=6)					
	Mean	**SD**	**Median**	**Q1**	**Q3**	**IQR**
AUC 0-t (ng*h/mL)	27217.16	35874.16	14680.90	4247.6	30964.50	26716.90
Cmax (ng/mL)	3093.26	2644.58	2230	935	5370	4435
T½α (h)	0.82	1.14	0.39	0.31	0.67	0.36
	Regimen 1: Non-responders (n=14)					
	Mean	**SD**	**Median**	**Q1**	**Q3**	**IQR**
AUC0-t (ng*h/mL)	36415.34	27817.43	30067.30	9955.2	57898.60	47943.40
Cmax (ng/mL)	4850.31	4017.83	3930	1827.5	7000	5172.5
T½α (h)	1.63	6.22	0.43	0.31	0.77	0.45
	Regimen 2: Responders (n=7)					
	Mean	**SD**	**Median**	**Q1**	**Q3**	**IQR**
AUC0-t (ng*h/mL)	42373.73	29422.60	37605.80	17328.70	57150.00	39821.30
Cmax (ng/mL)	5908.97	2980.33	5140	3410	7380	3970
T½α (h)	1.22	2.26	0.66	0.38	0.95	0.57
	Regimen 2: Non-responders (n=2)					
	Mean	**SD**	**Median**	**Q1**	**Q3**	**IQR**
AUC0-t (ng*h/mL)	21223.37	11552.67	15790.50	2932.55	28012.00	15079.45
Cmax (ng/mL)	3518.89	672.14	2740	2085	5215	3130
T½α (h)	1.51	3.02	0.49	0.37	0.75	0.38
	Regimen 3: Responders (n=1)					
	Mean	**SD**	**Median**	**Q1**	**Q3**	**IQR**
AUC0-t (ng*h/mL)	56051.02	88631.26	17248.55	14352.45	78629.75	64277.30
Cmax (ng/mL)	7003.33	374.75	6020	2957.5	952.5	7995
T½α (h)	1.28	0.79	1.47	0.39	2.07	1.68
	Regimen 3: Non-responders (n=8)					
	Mean	**SD**	**Median**	**Q1**	**Q3**	**IQR**
AUC0-t (ng*h/mL)	36958.50	19516.34	32625.30	22142.40	49225.05	27082.65
Cmax (ng/mL)	6055.76	2266.87	6060	4065	7580	3515
T½α (h)	1.87	2.24	1.04	0.56	2.08	1.51

Note: SD: Standard Deviation; Q1: First quartile; Q3: Third quartile; IQR: Interquartile Range; AUC0-t: Area Under the Curve; Cmax: Maximum (peak) serum concentration; Tmax: Time to maximum (peak) serum concentration; T½α: Distribution serum half-time and CL: Serum clearance.

Table 4: Pharmacokinetic parameters of responders and non-responders treated with MitoTam in phase Ib.

	Regimen 1: Responders RCC (n=2)					
	Mean	**SD**	**Median**	**Q1**	**Q3**	**IQR**
AUC0-t (ng*h/mL)	38 307.03	50 468.62	13 503.85	5489.4	68625.45	63136.05
Cmax (ng/mL)	3213.88	3208.41	1570	941.5	6560	5618.5
T½α (h)	1.02	1.09	0.56	0.38	1.2	0.82
	Regimen 1: Responders non-RCC (n=4)					
	Mean	**SD**	**Median**	**Q1**	**Q3**	**IQR**
AUC0-t (ng*h/mL)	21268.49	16483.64	19824.10	4274.68	32202.38	27927.70
Cmax (ng/mL)	3129.06	2341.53	3225	607	5195	4588
T½α (h)	0.66	1.08	0.34	0.24	0.58	0.34
	Regimen 2: Responders RCC (n=3)					
	Mean	**SD**	**Median**	**Q1**	**Q3**	**IQR**
AUC0-t (ng*h/mL)	41820.56	20982.20	49690.00	21111.25	57802.55	36691.30
Cmax (ng/mL)	6219.41	2671.45	5770	3955	8530	4575
T½α (h)	0.93	0.82	0.66	0.36	0.89	0.53
	Regimen 2: Responders non-RCC (n=4)					
	Mean	**SD**	**Median**	**Q1**	**Q3**	**IQR**
AUC0-t (ng*h/mL)	41255.45	34563.95	29365.90	14790.70	49680.00	34889.30
Cmax (ng/mL)	5338.64	3171.58	4480	2720	7302.5	4582.5
T½α (h)	1.44	2.87	0.57	0.41	1.08	0.67

Note: SD: Standard Deviation; Q1: First quartile; Q3: Third quartile; IQR: Interquartile Range; AUC0-t: Area Under the Curve; Cmax: Maximum (peak) serum concentration; Tmax: Time to maximum (peak) serum concentration and T½α: Distribution serum half-time.

Table 5: Pharmacokinetic parameters of responders with RCC and responders with other diagnoses treated with MitoTam in phase Ib.

Exposure-toxicity relationship

In phase I, the incidence and grade of the most frequent toxicities (i.e. hematological AEs and fever) increased with increasing dose of MitoTam [9]. Pharmacologically, AUC0-t, Cmax, T½α and CL were significantly prolonged in the 4.0, 5.0 and 6.0 mg/kg studies (Table 2). The mean AUC0-t was disproportionally greater in the 5.0 mg/kg studies compared with the 4.0 mg/kg study (47833 ng*h/mL (SD 36209) *vs.* 27421 ng*h/mL (SD 2487), respectively). The same pattern was seen for both Cmax (6395 ng/mL (SD 3773) *vs.* 3312 ng/mL (SD 824), respectively) and CL (142.52 mL/h/kg *vs.* 49.7 mL/h/kg, respectively). Only one patient was enrolled in the 6.0 mg/kg study. In addition to G1-G3 hematological AEs, this patient experienced a gastrointestinal toxicity (loss of appetite). The AUC0-t and Cmax were 119682.50 ng*h/mL and 15800 ng/mL, respectively, in this patient.

Similarly, in phase Ib, hematological and gastrointestinal toxicities (nausea, vomiting, diarrhea, loss of appetite, weight loss) occurred in about 20% of patients in regimen 2 and in 70%-80% of patients in regimen 3. T½α was significantly longer in regimen 3 than in regimen 2 (1.80 h (SD 2.11) *vs.* 1.28 h (SD 2.43), p=0.007). The difference in T½α was not due to the variability in protein blood levels between individual patients, because the mean serum total protein concentrations were not significantly different between regimens 2 and 3 (73.08 g/L (SD 2.83) *vs.* 72.80 g/L (SD 6.98), respectively, p=0.452). The mean serum albumin concentration in regimens 2 and 3 were 35.32 g/L (SD 3.15) and 35.30 g/L (SD 2.74), respectively (p=0.723). Thus, our preliminary hypothesis of depleted albumin binding and a greater free fraction of MitoTam in regimen 3 was not confirmed. Table 6 shows the total serum protein and albumin levels for all cohorts in phase Ib. Because there were no significant differences in AUC0-t, Cmax and CL, despite the different doses among these studies, we consider the 3.0 mg/kg dose to be optimal for further testing.

	Regimen 1: 1.0 mg/kg (n=20)					
	Mean	**SD**	**Median**	**Q1**	**Q3**	**IQR**
Serum total protein (g/L)	71	9.23	72.65	66.85	75.95	9.1
Serum albumin (g/L)	34.2	3.07	33.7	32.78	37.15	4.38
	Regimen 2: 3.0 mg/kg (n=9)					
	Mean	**SD**	**Median**	**Q1**	**Q3**	**IQR**
Serum total protein (g/L)	73.08	2.83	73.4	72.4	74.45	2.4
Serum albumin (g/L)	35.32	3.15	34	32.72	38.7	5.95
	Regimen 3: 4.0 mg/kg (n=9)					
	Mean	**SD**	**Median**	**Q1**	**Q3**	**IQR**
Serum total protein (g/L)	72.8	6.98	71.5	66.35	79.05	12.7
Serum albumin (g/L)	35.3	2.74	34.7	33.45	36.95	3.5

Note: SD: Standard Deviation; Q1: First quartile; Q3: Third quartile and IQR: Interquartile Range.

Table 6: Serum total protein and albumin levels in all cohorts in phase Ib.

Discussion

Here, we performed detailed analyses of the PK profile of MitoTam and subanalyses of the clinically relevant endpoints of MitoTam phase I/Ib trial from the perspective of PK findings. To expand on the main PK results, we performed additional analyses to reflect the two treatment schemas and clinico-pathological variables, including the toxicity of MitoTam. Our approach was based on comparative statistical analysis of the calculated PK parameters, the role of the treatment regimen, the number of treatment cycles, the dose of MitoTam, sex and baseline diagnosis. We paid special attention to whether the exceptional treatment outcomes in regimen 2 (CBR 78%) and in a virtual sub study of patients with RCC (CBR 83%) were statistically significant or a random finding. All of these variables were tested to better understand their impact on the PK of MitoTam.

The PK analysis showed a low extraction ratio and rapid distribution to the periphery. Most of the PK profiles indicated possible redistribution of MitoTam from the tissues or protein binding back into the serum, because secondary peaks in serum concentrations were occasionally observed. These secondary peaks were first observed in the 1.5 mg/kg study in phase I and subsequently observed in all studies in phase Ib. Preclinical bio-distribution studies in animals [8] showed that MitoTam mostly accumulated in the kidneys, myocardium, lungs and liver. Increased metabolism and high concentrations of the N-desmethyl MitoTam metabolite were observed in the liver and duodenum within 24 h post-dose, whereas the concentration in the kidneys increased steadily over a 1-week period, suggestive of accumulation rather than metabolization in this organ. The preclinical findings might help explain which tissues are the likely source of the secondary MitoTam peak. Overall, our clinical observations support the preclinical findings that MitoTam is excreted through the liver and bile ducts rather than *via* the kidneys.

MitoTam was detectable as long as 168 h after the start of the infusion, supporting the idea of a large Vz and high tissue affinity. We believe that the pig model used in preclinical studies [8] adequately addresses and explains the large volume of MitoTam distribution, however, clinically it is not feasible to confirm high tissue affinity since it is ethically unacceptable to take samples and evaluate PK parameters from patient tumors, liver and/or kidneys.

Regarding the study's primary objective to determine the optimal and safe dose for further testing we evaluated the relationship between the MitoTam dose and PK parameters. Elevated AUC0-t, Cmax, T½α and CL were recorded in the 4.0, 5.0 and 6.0 mg/kg studies in phase I and in regimen 3 of phase Ib. However, the differences in PK parameters between regimens 2 and 3 in phase Ib were generally not significant (AUC0-t, Cmax and CL) with the exception of T½α. The prolonged serum half-life T½α was not related to the serum total protein and albumin concentrations. Our hypothesis that the elevated T½α at doses above 4.0 mg/kg may be related to depleted albumin binding and a subsequent greater free fraction of MitoTam proved to be wrong. Rather, it seems that the significantly longer T½α at doses above 4.0 mg/kg is related to the already exhausted terminal elimination process, which correlates with our clinical observations. We can conclude that the dose of 3.0 mg/kg (in regimen 2) is optimal for further testing from clinical and pharmacological perspectives.

The AEs at the dose of 3.0 mg/kg were predominantly G1/2 anemia [9], a promising finding when compared to the safety profiles of other mitochondrial agents [2-6]. The risk of TE, which occurred in 13% of patients in phase Ib, may be related to the greater bio-distribution of

MitoTam in lung tissue, as observed in the preclinical model and the lipophilic properties of the drug. Nevertheless, risk factors such as prior history of TE disease, the malignancy itself and a long presence of an inadequate venous route (i.e. peripherally inserted central catheter) should be considered.

Regarding the efficacy of MitoTam, preclinical studies demonstrated high anticancer activity in several mouse models of cancer [12]. Our hypothesis that the CBR would be related to the PK parameters, primarily the AUC0-t and Cmax, in regimens 2 and 3, was not confirmed. We think this is due to the high Vz of MitoTam, the high permeability of MitoTam into cells and its high binding to tissue components. We thus conclude that the high CBR of MitoTam in patients with RCC is due to preferential accumulation in the kidneys. The number of patients whose tumors originated in the mesodermal layer is too small to conclude whether MitoTam is effective in mesodermal-layer-derived tumors other than RCC.

We searched for articles published in PubMed up to March 2023 and we found 88 studies in which cancers were targeted with mitochondrial inhibitors. Only 12 were active studies, almost exclusively in phases I or II. From this perspective, the successful antitumor effects of MitoTam observed in this trial hold great promise. The limitations of our study were discussed previously [9].

Some limitations, particularly the small number of participants, are inherent to phase I clinical trials. We are aware that the exposure-efficacy relationship is not well supported by data due to the small number of patients, resulting in confidence intervals for these comparisons being too wide and overlapping. The uneven representation of diagnoses between the study cohorts was due to the random recruitment of patients in the phase I trial, which was not powered to assess efficacy outcomes. The main technical limitation of our study was the use of two alternative treatment schemes in three regimens, which makes it difficult to compare the results among the patient groups. Therefore, we focused our PK analysis on the cohorts/regimens using a weekly dosing schedule. We believe that the results provide strong statistical evidence of clinical activity of MitoTam in tumors originating in the ME, which should be considered in future prospective phase II studies. Further confirmation of the clinical activity of MitoTam in patients with RCC is warranted.

Conclusion

We have demonstrated the safety and clinical activity of MitoTam from a pharmacological perspective. The PK parameters confirmed a two-compartment model with a large distribution of MitoTam into tissues and possible re-distribution back to the serum. The detailed analysis focused on the relationship between the PK of MitoTam and its toxicity and results support the previously recommended dose of 3.0 mg/kg with weekly administration for future studies. Patients with RCC showed greater exposure to MitoTam than other responders, which can be explained by its preferential accumulation in kidneys. Our data support further research of drug candidates targeting mitochondria.

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Authors' Contributions

Zuzana Bielcikova: Conceptualization; Investigation; Methodology; Writing-original draft; Writing-review and editing. Olga Bartosova: Conceptualization; Formal analysis; Methodology; Writing-review and editing. Jan Stursa: Investigation; Methodology; Visualization; Writing-review and editing. Michal Pesta: Data curation; Formal analysis; Writing-review and editing. Jiri Neuzil: Funding Acquisition; Supervision; Writing-review and editing. Ondrej Slanar: Project Administration; Validation; Supervision; Writing-review and editing. Irena Stenglova Netikova: Methodology; Validation; Supervision; Writing-review and editing. Miroslava Bursova: Investigation; Validation; Writing-review & editing. Lukas Werner: Investigation; Methodology; Supervision; Writing-review and editing.

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Competing Interests

Jiri Neuzil, Jan Stursa and Lukas Werner are owners of MitoTax (společnost s ručením omezeným) that co-authors the MitoTam intellectual property. The remaining authors declare that they have no conflict of interest.

Ethical Approval

The MitoTam-01 study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethical Review Committee of the General Faculty Hospital, Charles University.

Consent to Participate

All patients signed written informed consent before undergoing any study-related procedure.

Data Availability

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

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