Inhibitor pre-incubation for transporter studies– an artefact of the *in vitro* test system?

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Who We Are and What We Do

Established in 1999, Cyprotex Limited was acquired by Evotec AG (www.evotec.com) in 2016

Specialize in *in vitro* and *in silico* ADME-Tox services:

- *in vitro* ADME screening
- regulatory *in vitro* ADME
- DDI studies (clinical/preclinical)
- *in vitro* human and animal toxicity modeling
- PBPK/QSAR modelling

Culture of innovation and scientific enquiry

- Strong focus on R&D
- Collaborative
- Knowledgeable & Experienced
- Solutions-focussed

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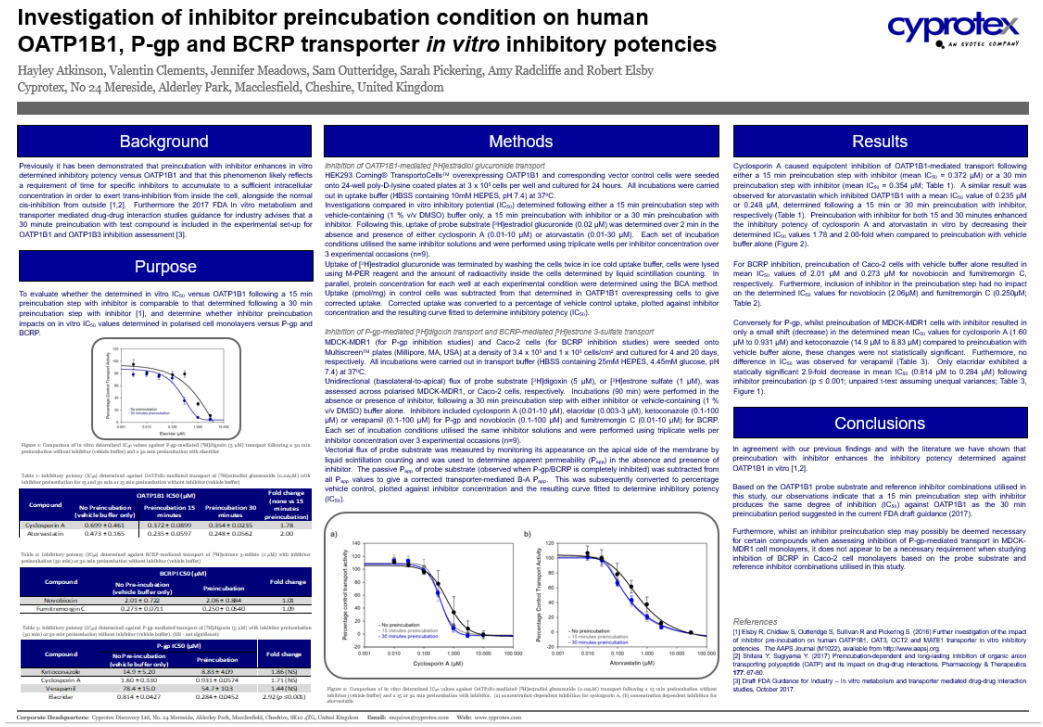
Current position and aim of research



Investigation of inhibitor preincubation condition on human OATP1B1, P-gp and BCRP transporter *in vitro* inhibitory potencies

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Background

Previously it has been demonstrated that preincubation with inhibitor enhances in vitro determined inhibitory potency versus OATP1B1 and that this phenomenon likely reflects a requirement of time for specific inhibitors to accumulate to a sufficient intracellular concentration in order to exert trans-inhibition from inside the cell, alongside the normal cis-inhibition from outside [1,2]. Furthermore the 2017 FDA In vitro metabolism and transporter mediated drug-drug interaction studies guidance for industry advises that a 30 minute preincubation with test compound is included in the experimental set-up for OATP1B1 and OATP1B3 inhibition assessment [3].

Purpose

To evaluate whether the determined in vitro IC_{50} versus OATP1B1 following a 15 min preincubation step with inhibitor is comparable to that determined following a 30 min preincubation step with inhibitor [1], and determine whether inhibitor preincubation impacts on in vitro IC_{50} values determined in polarised cell monolayers versus P-gp and BCRP.

Methods

Inhibition of OATP1B1-mediated [³H]estradiol glucuronide transport

HEK293 Corning® TransportoCells™ overexpressing OATP1B1 and corresponding vector control cells were seeded onto 24-well poly-D-lysine coated plates at 3 x 10^5 cells per well and cultured for 24 hours. All incubations were carried out in uptake buffer (HBSS containing 10mM HEPES, pH 7.4) at 37ºC.

Investigations compared in vitro inhibitory potential (IC_{50}) determined following either a 15 min preincubation step with vehicle-containing (1 % v/v DMSO) buffer only, a 15 min preincubation with inhibitor or a 30 min preincubation with inhibitor. Following this, uptake of probe substrate [³H]estradiol glucuronide (0.02 µM) was determined over 2 min in the absence and presence of either cyclosporin A (0.01-10 µM) or atorvastatin (0.01-30 µM). Each set of incubation conditions utilised the same inhibitor solutions and were performed using triplicate wells per inhibitor concentration over 3 experimental occasions (n=9).

Uptake of [³H]estradiol glucuronide was terminated by washing the cells twice in ice cold uptake buffer, cells were lysed using M-PER reagent and the amount of radioactivity inside the cells determined by liquid scintillation counting. In parallel, protein concentration for each well at each experimental condition were determined using the BCA method. Uptake (pmol/mg) in control cells was subtracted from that determined in OATP1B1 overexpressing cells to give corrected uptake. Corrected uptake was converted to a percentage of vehicle control uptake, plotted against inhibitor concentration and the resulting curve fitted to determine inhibitory potency (IC_{50}).

Inhibition of P-gp-mediated [³H]digoxin transport and BCRP-mediated [³H]estrone 3-sulfate transport

MDCK-MDR1 (for P-gp inhibition studies) and Caco-2 cells (for BCRP inhibition studies) were seeded onto Multiscreen™ plates (Millipore, MA, USA) at a density of 3.4 x 10^5 and 1 x 10^5 cells/cm² and cultured for 4 and 20 days, respectively. All incubations were carried out in transport buffer (HBSS containing 25mM HEPES, 4.45mM glucose, pH 7.4) at 37ºC.

Unidirectional (basolateral-to-apical) flux of probe substrate [³H]digoxin (5 µM), or [³H]estrone sulfate (1 µM), was assessed across polarised MDCK-MDR1, or Caco-2 cells, respectively. Incubations (90 min) were performed in the absence or presence of inhibitor, following a 30 min preincubation step with either inhibitor or vehicle-containing (1 % v/v DMSO) buffer alone. Inhibitors included cyclosporin A (0.01-10 µM), elacridar (0.003-3 µM), ketoconazole (0.1-100 µM) or verapamil (0.1-100 µM) for P-gp and novobiocin (0.1-100 µM) and fumitremorgin C (0.01-10 µM) for BCRP. Each set of incubation conditions utilised the same inhibitor solutions and were performed using triplicate wells per inhibitor concentration over 3 experimental occasions (n=9).

Vectorial flux of probe substrate was measured by monitoring its appearance on the apical side of the membrane by liquid scintillation counting and was used to determine apparent permeability (P_{app}) in the absence and presence of inhibitor. The passive P_{app} of probe substrate (observed when P-gp/BCRP is completely inhibited) was subtracted from all P_{app} values to give a corrected transporter-mediated B-A P_{app}. This was subsequently converted to percentage vehicle control, plotted against inhibitor concentration and the resulting curve fitted to determine inhibitory potency (IC_{50}).

Results

Cyclosporin A caused equipotent inhibition of OATP1B1-mediated transport following either a 15 min preincubation step with inhibitor (mean IC_{50} = 0.372 µM) or a 30 min preincubation step with inhibitor (mean IC_{50} = 0.354 µM; Table 1). A similar result was observed for atorvastatin which inhibited OATP1B1 with a mean IC_{50} value of 0.235 µM or 0.248 µM, determined following a 15 min or 30 min preincubation with inhibitor, respectively (Table 1). Preincubation with inhibitor for both 15 and 30 minutes enhanced the inhibitory potency of cyclosporin A and atorvastatin in vitro by decreasing their determined IC_{50} values 1.78 and 2.00-fold when compared to preincubation with vehicle buffer alone (Figure 2).

For BCRP inhibition, preincubation of Caco-2 cells with vehicle buffer alone resulted in mean IC_{50} values of 2.01 µM and 0.273 µM for novobiocin and fumitremorgin C, respectively. Furthermore, inclusion of inhibitor in the preincubation step had no impact on the determined IC_{50} values for novobiocin (2.06µM) and fumitremorgin C (0.250µM; Table 2).

Conversely for P-gp, whilst preincubation of MDCK-MDR1 cells with inhibitor resulted in only a small shift (decrease) in the determined mean IC_{50} values for cyclosporin A (1.60 µM to 0.931 µM) and ketoconazole (14.9 µM to 8.83 µM) compared to preincubation with vehicle buffer alone, these changes were not statistically significant. Furthermore, no difference in IC_{50} was observed for verapamil (Table 3). Only elacridar exhibited a statistically significant 2.9-fold decrease in mean IC_{50} (0.814 µM to 0.284 µM) following inhibitor preincubation ($p \leq 0.001$; unpaired t-test assuming unequal variances; Table 3, Figure 1).

Conclusions

In agreement with our previous findings and with the literature we have shown that preincubation with inhibitor enhances the inhibitory potency determined against OATP1B1 in vitro [1,2].

Based on the OATP1B1 probe substrate and reference inhibitor combinations utilised in this study, our observations indicate that a 15 min preincubation step with inhibitor produces the same degree of inhibition (IC_{50}) against OATP1B1 as the 30 min preincubation period suggested in the current FDA draft guidance (2017).

Furthermore, whilst an inhibitor preincubation step may possibly be deemed necessary for certain compounds when assessing inhibition of P-gp-mediated transport in MDCK-MDR1 cell monolayers, it does not appear to be a necessary requirement when studying inhibition of BCRP in Caco-2 cell monolayers based on the probe substrate and reference inhibitor combinations utilised in this study.

References

[1] Elsby R, Chidlaw S, Outteridge S, Sullivan R and Pickering S. (2016) Further investigation of the impact of inhibitor pre-incubation on human OATP1B1, OAT3, OCT2 and MATE1 transporter in vitro inhibitory potencies. The AAPS Journal (M1022), available from http://www.aaps.org
[2] Shitara Y, Sugiyama Y. (2017) Preincubation-dependent and long-lasting inhibition of organic anion transporting polypeptide (OATP) and its impact on drug-drug interactions. Pharmacology & Therapeutics 177: 67-90
[3] Draft FDA Guidance for Industry – In vitro metabolism and transporter mediated drug-drug interaction studies, October 2017.

Current Position:

- Recommended transporters for inhibition studies*:* P-gp, BCRP, OATP1B1, OATP1B3, OAT1, OAT3, OCT2, MATE1 and MATE2K (BSEP and OCT1 also considered in EMA guidance)
- Recent FDA guidance indicates 30 min pre-incubation with inhibitor required for OATP1B1 and OATP1B3

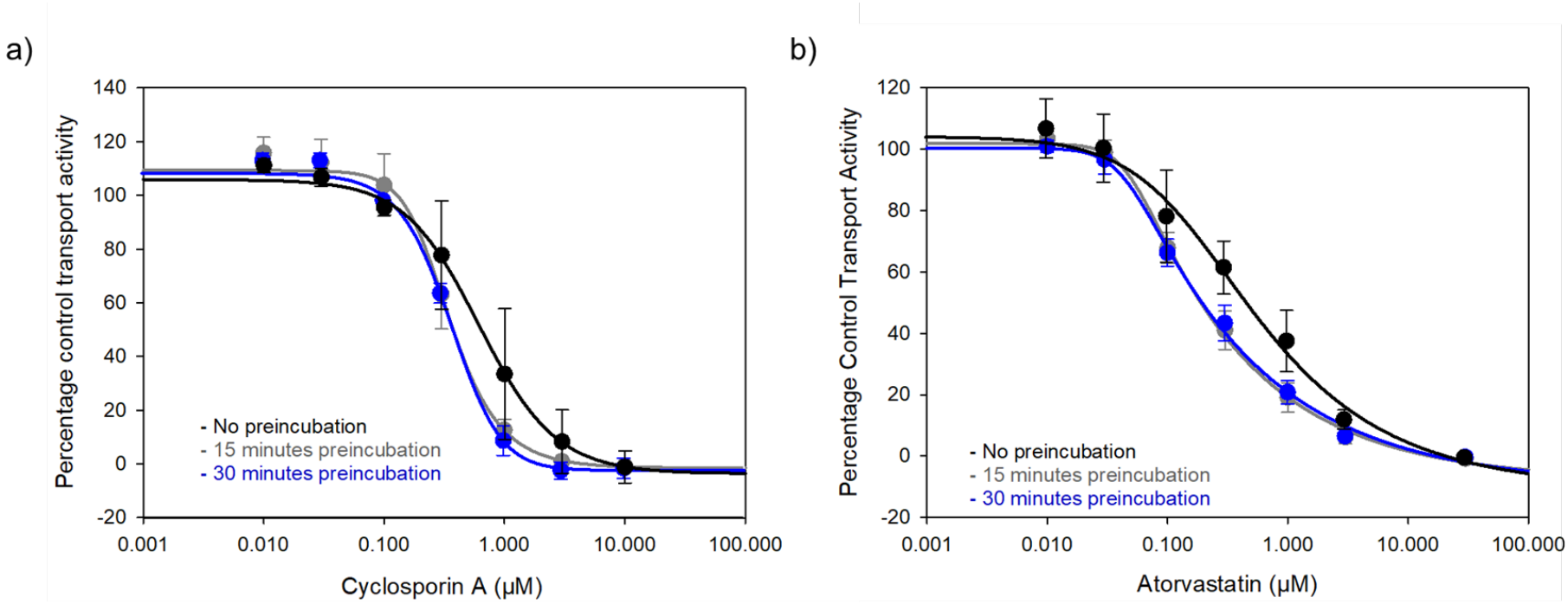
Aim of Research

- Time dependency of inhibitory effect
- Impact of pre-incubation on *in vitro* IC_{50} values for polarised cell lines such as P-gp and BCRP

Time dependency of pre-incubation

15 min vs 30 min

- No difference in 15 min pre-incubation vs 30 min pre-incubation period for OATP1B1
- Reduction in pre-incubation time has benefits in terms of unstable compounds and throughput efficiency



Transporter dependency on pre-incubation

Polarised cells

- Inhibitor pre-incubation may also be necessary for P-gp-mediated transport in MDR1-MDCK cells where effects are compound-dependent
- BCRP in Caco-2 cells not affected by pre-incubation

Compound	P-gp IC50 (µM)		Fold change
	No Pre-incubation (vehicle buffer only)	Preincubation	
Ketoconazole	14.9 ± 5.20	8.83 ± 4.09	1.86 (NS)
Cyclosporin A	1.60 ± 0.330	0.931 ± 0.0574	1.71 (NS)
Verapamil	78.4 ± 15.0	54.7 ± 10.3	1.44 (NS)
Elacridar	0.814 ± 0.0427	0.284 ± 0.0452	2.92 ($p \leq 0.001$)

Compound	BCRP IC50 (µM)		Fold change
	No Pre-incubation (vehicle buffer only)	Preincubation	
Novobiocin	2.01 ± 0.722	2.06 ± 0.884	1.01
Fumitremorgin C	0.273 ± 0.0711	0.250 ± 0.0540	1.09

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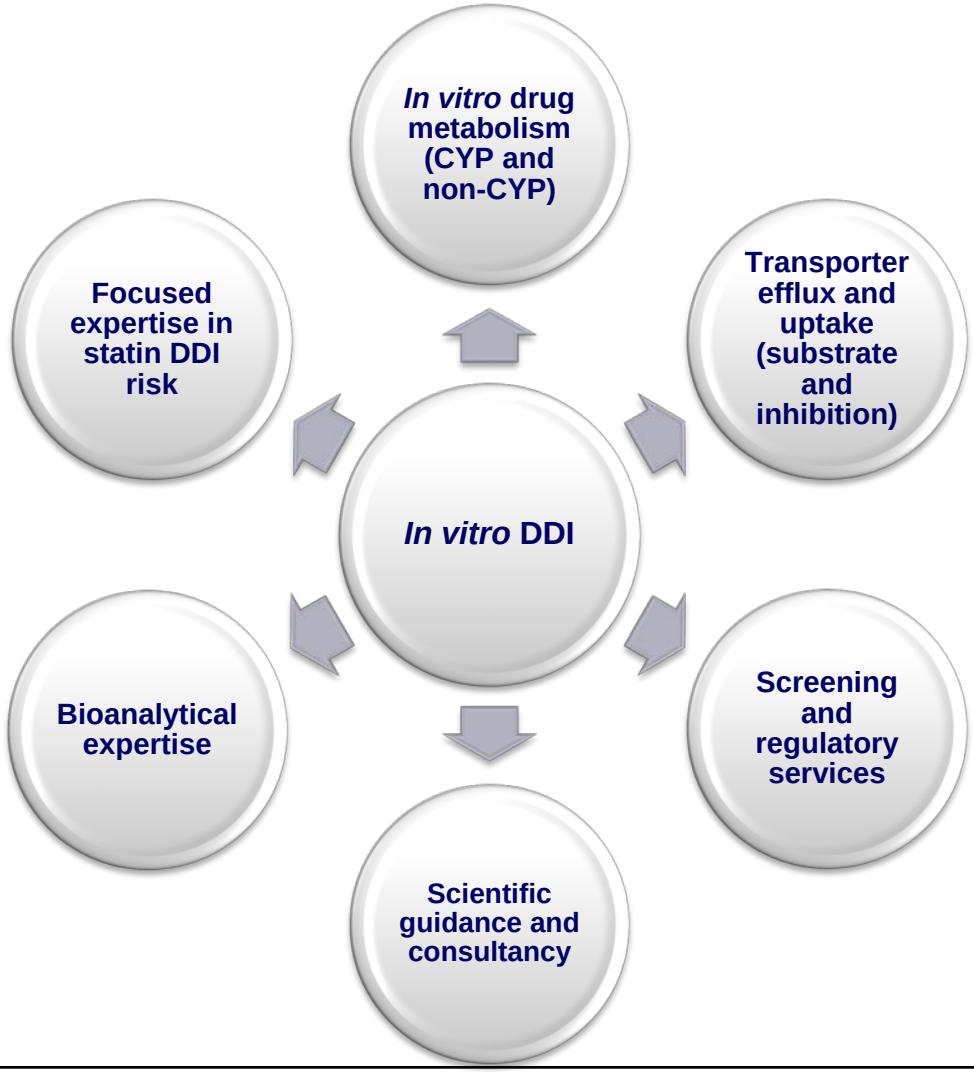
Capabilities and expertise in *in vitro* DDI studies

- *In vitro* drug metabolism (CYP and non-CYP) and transporters (efflux and uptake)
- Scientific guidance and consultancy -- expertise in the design, implementation and interpretation of DDI studies
- Both screening and regulatory services to support IND and NDA submission
- Bioanalytical expertise
- IVIV extrapolation: focused expertise in understanding statin DDI risk (predicted maximal theoretical AUC change) using mechanistic static approach



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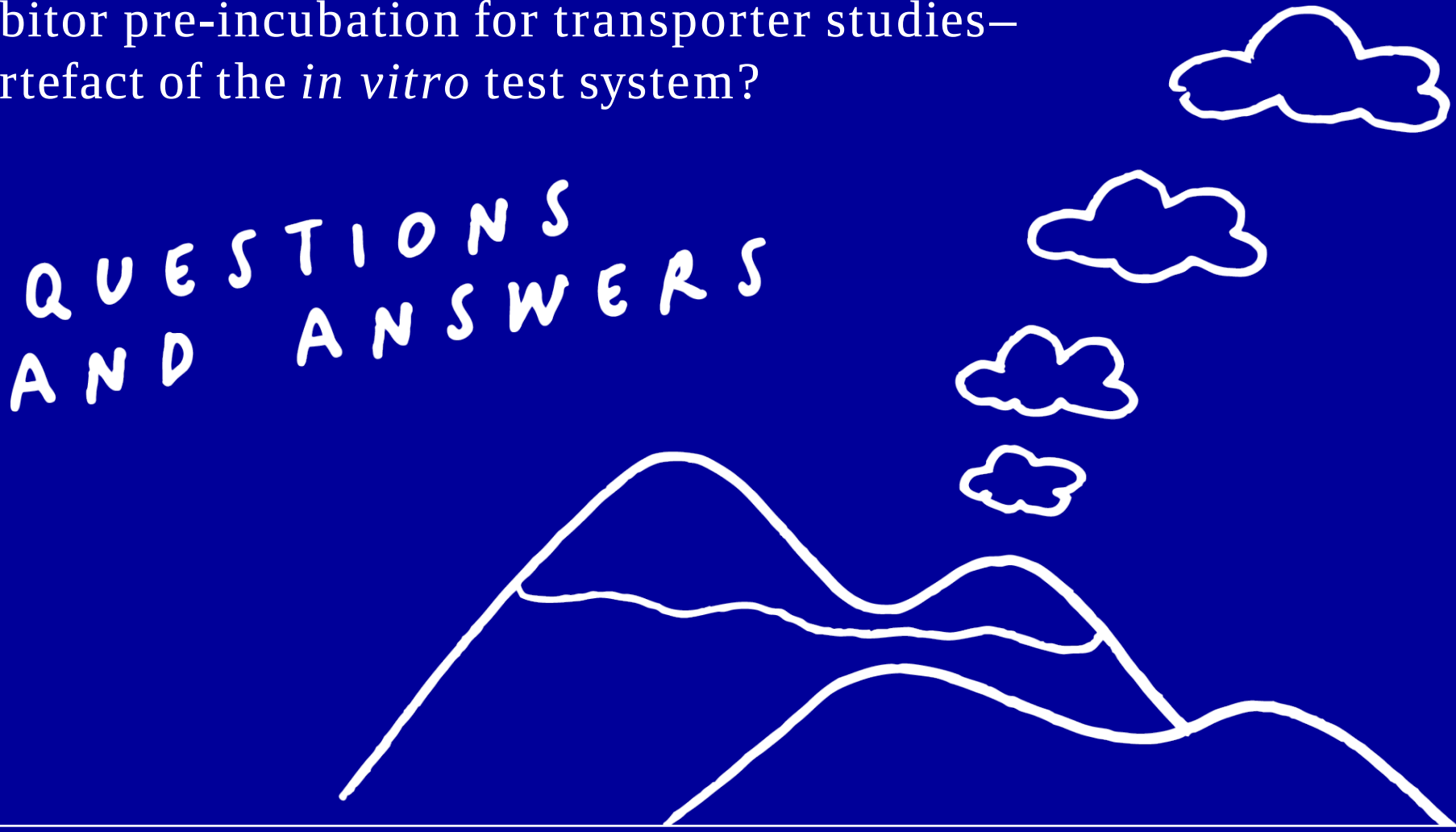

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