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Controlled release of MT-1207 using a novel gastroretentive bilayer system comprised of hydrophilic and hydrophobic polymers

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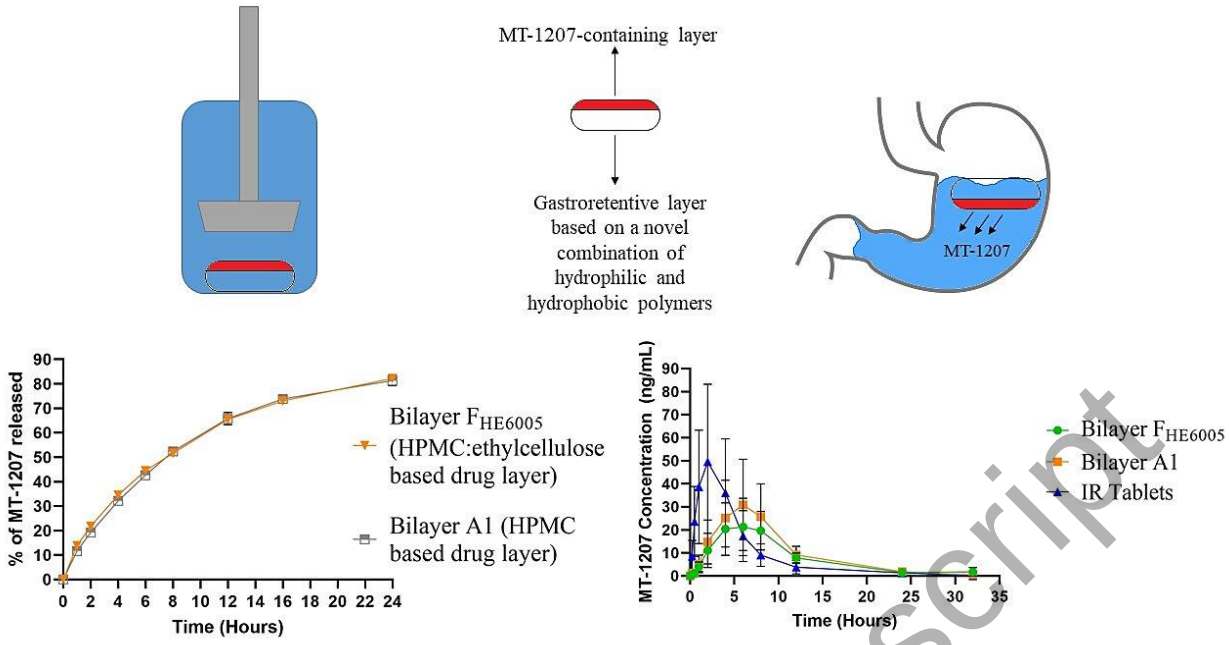
Controlled release of MT-1207 using a novel gastroretentive bilayer system comprised of hydrophilic and hydrophobic polymers

Abstract

In the present study, novel gastroretentive bilayer tablets were developed that are promising for the once-a-day oral delivery of the drug candidate MT-1207. The gastroretentive layer consisted of a combination of hydrophilic and hydrophobic polymers, namely polyethylene oxide and Kollidon® SR. A factorial experiment was conducted, and the results revealed a non-effervescent gastroretentive layer that, unlike most gastroretentive layers reported in the literature, was easy to prepare, and provided immediate tablet buoyancy (mean floating lag time of 1.5 seconds) that lasted over 24 hours in fasted state simulated gastric fluid (FaSSGF) pH 1.6, irrespective of the drug layer, thereby allowing a 24-hour sustained release of MT-1207 from the drug layer of the tablets. Furthermore, during *in vitro* buoyancy testing of the optimised bilayer tablets in media of different pH values (1.0, 3.0, 6.0), the significant difference (one-way ANOVA, $p < 0.001$) between the respective total floating times indicated that stomach pH effects on tablet buoyancy are important to be considered during the development of non-effervescent gastroretentive formulations and the choice of dosing regimen. To the best of our knowledge, this has not been reported before, and it should probably be factored in when designing dosing regimens. Finally, a pharmacokinetic study in Beagle dogs indicated a successful *in vivo* 24-hour sustained release of MT-1207 from the optimised gastroretentive bilayer tablet formulations with the drug plasma concentration remaining above the estimated minimum effective concentration of 1 ng/mL at the 24-hour timepoint and also demonstrated the gastroretentive capabilities of the hydrophilic and hydrophobic polymer combination. The optimised formulations will be forwarded to clinical development.

Keywords: MT-1207; gastroretentive tablets; bilayer tablets; novel gastroretentive layer; pH effect; buoyancy

Graphical abstract



1 Introduction

Hypertension is the most common cardiovascular disease [1]. Globally, approximately 1.28 billion adults aged 30 − 79 years have hypertension with only about 21 % of them having it under control [2]. A variety of parameters influence the incidence and severity of hypertension, including race, age, and education [3-5]. Hypertension is the leading preventable risk factor for premature death and disability globally [6]. The elevated blood pressure linked to this disease is associated with a high risk of end-organ damage to the kidney, heart and brain, as seen from epidemiologic studies. Both systolic and diastolic hypertension are related to end-organ damage [7, 8].

Different drug classes are used in hypertension treatment, including angiotensin receptor blockers, angiotensin-converting enzyme inhibitors, beta blockers, calcium channel blockers, diuretics, and alpha-receptor antagonists [9-11]. In recent years, the paradigm regarding the start of antihypertensive therapy has shifted from monotherapy to drug combinations since they have proved more effective in achieving management of hypertension [12]. The use of many different drugs targeting different receptors is called polypharmacy (the concurrent use of multiple medications by a patient) [9, 13]. However, the consequent need to take many separate medicines can lead to reduced patient compliance.

MT-1207 hydrochloride is a new drug candidate that is currently in phase II clinical trials, possessing antihypertensive properties derived from combinatory pharmacological actions. It has been demonstrated strongly that MT-1207 is an antagonist towards calcium channels, adrenergic α_{1A}, α_{1B}, α_{1D} and serotonin (5-Hydroxytryptamine) 5-HT_{2A} receptors simultaneously. Therefore, the mechanism underlying the hypotensive effect of MT-1207 is at least a combination of vasodilation (through α receptors and vascular smooth muscle relaxation via the calcium channel) and bradycardia (through 5-HT_{2A} receptors) [14, 15]. The safety and tolerability of MT-1207 in healthy humans have been demonstrated in the dose range of 5 – 40 mg, alongside an antihypertensive effect in the dose range of 10 – 40 mg. Since a multi-target therapeutic strategy is desired for the successful management of hypertension, an agent targeting multiple receptors, such as MT-1207, could be a promising choice for the treatment of hypertension. Therefore, MT-1207 is currently being tested in phase Ib/IIa clinical trials in hypertensive patients at a dose range of 10 – 40 mg with a suggested twice-daily dosing regimen [15]. Chemically, MT-1207 possesses poor aqueous solubility (0.03 mg/mL at 37 °C) and strong lipophilicity (logP of 4.15) with a pK_a value of 7.31.

Given that hypertension is a chronic condition, a steady-state concentration of the drug(s) ideally should be secured throughout the dosing regimen [16]. Controlled-release formulations are usually developed to

achieve drug release in the desired gastrointestinal tract site at a predetermined rate. In our previous study, an MT-1207 sustained-release HPMC tablet formulation (formulation A1) was developed as a promising choice for the once-a-day oral delivery of the drug [17]. Here, we further optimised the sustained-release formulation by expanding the traditional single-layer HPMC matrix tablet approach with a gastroretentive drug delivery system (GRDDS) element.

GRDDS are delivery systems that can effectively be retained in the stomach and release drugs locally. Their utilisation is associated with advantages that include prolonged gastric residence time (GRT) of dosage forms in the stomach up to several hours which, in turn, may result in an efficient targeted delivery in the stomach and/or increased therapeutic efficacy by improving drug absorption. GRDDS can be used as controlled-release platforms to quantitatively deliver drugs to the desired absorption site for an extended period at a certain rate [18-20]. Their application can be beneficial for drugs, such as MT-1207, with a narrow absorption window in the proximal small intestine, low solubility and/or stability at alkaline intestinal pH, short half-life, plasma level fluctuations, as well as drugs aimed at acting locally in the stomach and upper part of the intestine for eradication of *Helicobacter pylori* [21-30].

In non-effervescent floating systems, highly swellable cellulose derivatives or gel-forming polymers are frequently used. Non-effervescent systems include hydrodynamically balanced systems (HBS), single- and double-layer floating tablets, and microballoons/hollow microspheres. Significant advantages of non-effervescent floating systems over effervescent formulations include the non-dependence of the floating mechanism on gastric pH which is variable and can be problematic in patients with achlorhydria, and the fact that the stability of acid- or base-labile drugs is secured through the exclusion of a gas-generating agent [21]. However, to date, no study has investigated the potential effect of pH on the duration of the buoyancy of non-effervescent gastroretentive systems.

To achieve efficient floatation and subsequent gastric retention, usually, hydrophilic polymers are used [21, 26, 31-33]. Gastroretentive tablets should have as little floating lag time as possible to prevent premature evacuation from the stomach. Their low density is key towards achieving rapid floatation, and this can be secured via the inclusion of low-density materials in the formulation, as well as the application of low tablet compression force during tablet preparation that allows for higher tablet porosity and, hence, reduced tablet density. The higher tablet porosity can also contribute to more rapid polymer wetting and swelling that will further reduce the density of the dosage form [26]. However, in a recent study, a hydrophobic polymer, Kollidon® SR, was used along with camphor in the gastroretentive layer of bilayer tablets. Kollidon® SR has a

low density and its hydrophobic nature contributed towards high wet strength of the layer, while the camphor sublimation further reduced the tablet density. As a result, the tablets prepared in that study successfully floated over a 12-hour time period *in vitro*, even at a dissolution apparatus paddle rotation rate of 200 rpm [34].

Both hydrophilic and hydrophobic polymers have proved effective in achieving gastric retention, especially when combined with gas-generating or sublimating agents. However, to the best of our knowledge, the application of a combination of hydrophilic and hydrophobic polymers for gastric retention purposes in gastroretentive tablets without the use of gas-generating or sublimating agents has not been reported. The swelling properties of hydrophilic polymers, combined with a low-density hydrophobic polymer, the presence of which will strengthen the forming gel, could eliminate the need for gas-generating or sublimating agents, and result in a gastroretentive formulation that would be easy to prepare and would be able to be retained in the stomach long enough to serve as a once-a-day controlled-release formulation, whilst withstanding the destructive forces experienced in the stomach.

In the present study, non-effervescent bilayer tablets containing MT-1207 were successfully prepared. The bilayer tablet development was divided into two parts: gastroretentive layer development and optimisation, and drug layer optimisation. The two major components of the gastroretentive layer were PEO and Kollidon® SR. These materials have been used in different studies for the preparation of gastroretentive formulations [21, 24, 34, 35] but not together in a combination. The low density and swelling capability of PEO render it a good choice for the preparation of floating systems. The low density of Kollidon® SR could further contribute to the floatation of gastroretentive systems, while its hydrophobic nature could ensure a more controlled hydration of the layer and a higher wet strength that would ensure its structural integrity *in vitro* and *in vivo*. Therefore, the combination of these two polymers in the gastroretentive layer was deemed promising. In the drug layer, ethylcellulose was incorporated as an additional release retardant to further sustain the release of MT-1207 from the tablets, whilst maintaining a sufficiently high wet strength throughout 24 hours of *in vitro* release. Furthermore, the *in vitro* buoyancy of the gastroretentive bilayer tablets was examined in media of different pH values. Based on the experimental findings, the pharmacokinetic study of the optimised bilayer tablets was conducted in Beagle dogs during the fed state.

2 Materials and Methods

2.1 Materials

MT-1207 hydrochloride reference standard and micronised MT-1207 hydrochloride:colloidal silicon dioxide mixture with a mass ratio of 5:1 were donated by Shenyang Haiwang Biotechnology Co., Ltd (Shenyang, China). Polyethylene oxide average molecular weight 8,000,000 (8M), Kollidon® SR, spray-dried lactose, polyvinylpyrrolidone K30 (PVP K30), hydroxypropyl methylcellulose average molecular weight 90,000 (K15M), hydroxypropyl methylcellulose average molecular weight 120,000 (K100M), colloidal silicon dioxide, magnesium stearate, sodium phosphate monobasic dihydrate and sodium acetate anhydrous were purchased from Sigma-Aldrich (Gillingham, United Kingdom). Alpha-D-lactose monohydrate, ethylcellulose 10 cps, iron (III) oxide, hydrochloric acid 37 %, acetic acid glacial, sodium hydroxide pellets, orthophosphoric acid, methanol HPLC grade and propan-2-ol HPLC grade were purchased from Fisher Scientific (Loughborough, United Kingdom).

2.2 HPLC-UV method for the quantitation of MT-1207 in vitro release samples

The HPLC-UV method conditions that were used for the quantitation of MT-1207 *in vitro* release samples were the same as the ones reported in our previous study, except for the range of the calibration curve [17]. In brief, a Dionex Ultimate 3000 HPLC system was used (Thermo Scientific®, United Kingdom) with an ACE® Generix 3 C18, 150 × 4.6 mm, 3 µm column. The mobile phase consisted of 0.1 M sodium acetate buffer pH 4.0 and methanol at a ratio of 40:60 (% v/v). The mobile phase flow rate was set at 0.7 mL/min. The UV wavelength was set at 230 nm. The injection volume was 20 µL. The retention time of MT-1207 was approximately 5 min (Figure S1, Supplementary material). The quantitation of MT-1207 samples was carried out using a calibration curve which covered a concentration range of 1 – 100 µg/mL (Figure S2, Supplementary material). The HPLC method was validated in terms of linearity, the limit of detection (LOD) and the limit of quantitation (LOQ) as per the International Council for Harmonisation (ICH) and FDA guidelines. The method LOD was 0.1 µg/mL and the LOQ was 0.3 µg/mL. The validation of the HPLC method for specificity, accuracy, intra-day and inter-day precision was carried out along with that of the dissolution method.

2.3 Flowability characterisation of the tablet blends

The tablet blend flowability was assessed in terms of bulk/tapped density, compressibility (Carr) index and Hausner ratio. Bulk/tapped density testing was carried out using tapped density tester (Copley®, Nottingham, United Kingdom) to determine the flow properties of the different powder mixtures as per USP <616> [36].

2.4 Preparation of gastroretentive bilayer tablets

For the gastroretentive layer, the appropriate amounts of PEO 8M, Kollidon® SR, spray-dried lactose, HPMC K100M and colloidal silicon dioxide were each time weighed and mixed manually for 15 minutes. Then, magnesium stearate was added to the powder mixture and mixing was carried out for 5 minutes.

The preparation of the drug sustained-release layer included a wet granulation step and was conducted following the same procedure as the preparation of the single-layer sustained-release tablets reported in our previous paper [17].

A Piccola rotary tablet press machine (Riva®, Argentina) was used for the compression process which was carried out in two steps. The gastroretentive powder mixture was first added into the die manually and a pre-compression step was applied, followed by the addition of the granulated drug layer on top of the pre-compressed gastroretentive layer. Compression was then carried out to prepare the bilayer tablets using compression force values of 5 kN, 7 kN, 7.5 kN or 10 kN. 13 mm flat-faced round and 17 × 9 mm concave oblong punches and dies were used during the compression process, each time depending on the tablet weight.

Table 1 lists the different drug layer formulas that were prepared and characterised in the present study.

Table 1: Compositions of the bilayer tablet ethylcellulose-containing drug layers (reported as mass in milligrams).

Components (mg)	F_{HE7035}	F_{HE7030}	F_{HE6030}	F_{HE6020}	F_{HE5020}	F_{HE6015}	F_{HE6010}	F_{HE6005}	F_{HE6003}	F_{HE6001}
MT-1207 Hydrochloride	30	30	30	30	30	30	30	30	30	30
Alpha-D-lactose monohydrate	45	45	55	60	70	65	70	65	67	69
Polyvinylpyrrolidone K30	0	5	5	10	10	10	10	20	20	20
HPMC K15M	70	70	60	60	50	60	60	60	60	60
Ethylcellulose 10 cps	35	30	30	20	20	15	10	5	3	1
Iron (III) oxide	1	1	1	1	1	1	1	1	1	1
Colloidal silicon dioxide	6	6	6	6	6	6	6	6	6	6
Magnesium stearate	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Total layer weight	**187.5**	**187.5**	**187.5**	**187.5**	**187.5**	**187.5**	**187.5**	**187.5**	**187.5**	**187.5**

2.5 Design of the factorial experiment for the gastroretentive layer

A face-centred central composite design was employed for the gastroretentive layer factorial experiment to assess the impact of the factor alterations on the *in vitro* buoyancy behaviour of the gastroretentive bilayer tablets. Based on preliminary experiments, the factors that were selected for the experiment were: the amount of PEO (mg) (X_1), the % of spray-dried lactose (SDL) (X_2) and the compression force (X_3). The percentages of Kollidon® SR and HPMC K100M in the gastroretentive layer were maintained stable and were selected based on preliminary experiment findings. In the experiment setup, each factor was examined over three levels (-1, 0, 1). Therefore, 27 different combinations were prepared and characterised (Table 2). The investigated dependent variables of the experiment were the floating lag time (the time taken for the tablets to start floating) and total floating time (the total time during which the tablets remained buoyant and were able to re-surface after application of vertical pressure using a spatula) of the bilayer tablets.

Table 2: Experimental layout of the gastroretentive layer factorial experiment.

Formulation	PEO 8M Amount (mg)	% of SDL	Compression Force (kN)	Kollidon® SR (mg)	HPMC K100M (mg)	Drug layer (mg)	Gastroretentive layer (mg)	**Total tablet weight (mg)**
1	480	7	7.5	290	20.5	187.5	850.5	**1038**
2	480	0	7.5	256	18.2	187.5	757.8	**945.3**
3	480	7	5	290	20.5	187.5	850.5	**1038**
4	480	0	5	256	18.2	187.5	757.8	**945.3**
5	480	7	10	290	20.5	187.5	850.5	**1038**
6	480	0	10	256	18.2	187.5	757.8	**945.3**
7	320	7	7.5	193.8	13.7	187.5	570	**757.5**
8	320	0	7.5	173.4	12.2	187.5	507.8	**695.3**
9	320	7	5	193.8	13.7	187.5	570	**757.5**
10	320	0	5	173.4	12.2	187.5	507.8	**695.3**
11	320	7	10	193.8	13.7	187.5	570	**757.5**
12	320	0	10	173.4	12.2	187.5	507.8	**695.3**
13	160	7	7.5	95.2	6.7	187.5	282.7	**470.2**
14	160	0	7.5	85.7	6	187.5	252.9	**440.4**
15	160	7	5	95.2	6.7	187.5	282.7	**470.2**
16	160	0	5	85.7	6	187.5	252.9	**440.4**
17	160	7	10	95.2	6.7	187.5	282.7	**470.2**
18	160	0	10	85.7	6	187.5	252.9	**440.4**
19	480	3.5	7.5	272	19.2	187.5	802.8	**990.3**
20	320	3.5	7.5	181.3	12.8	187.5	535.2	**722.7**
21	160	3.5	5	90.7	6.4	187.5	267.7	**455.2**
22	480	3.5	5	272	19.2	187.5	802.8	**990.3**
23	320	3.5	5	181.3	12.8	187.5	535.2	**722.7**
24	480	3.5	10	272	19.2	187.5	802.8	**990.3**
25	320	3.5	10	181.3	12.8	187.5	535.2	**722.7**
26	160	3.5	7.5	90.7	6.4	187.5	267.7	**455.2**
27	160	3.5	10	90.7	6.4	187.5	267.7	**455.2**

SigmaPlot® 13 software was used to analyse the data generated from the testing of the different gastroretentive layer combinations. Polynomial models were generated for both dependent variables. The best-fitting model was selected based on the R^2 and adjusted R^2 values. Also, the selected optimised gastroretentive layer was tested for its *in vitro* buoyancy behaviour, and its floating lag time and total floating time experimental values were compared to the ones predicted by the models to further assess the suitability of the models to

accurately predict the dependent variable values for different gastroretentive layer combinations. Furthermore, the influence of factors and factor interactions on the dependent variables was evaluated by ANOVA and the significance of their effect on the variables was assessed based on the calculated *p*-values.

The following mathematical Equation 1 was employed to describe mathematically the relationship between each of the dependent variables and the different factors, and the effect of the latter on the former:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 \quad (1)$$

where Y is the dependent variable, b_0 is the intercept and the other b_i values are constants describing the relationship between Y and the different factors (X_1, X_2, X_3), as well as the effect of changes occurring in two factors simultaneously. X_i^2 terms refer to the factor quadratic effect that has been included in the model for non-linear correlations. After the model equations for each of the dependent variables were generated and the effects of the factors and their interactions determined, an optimised gastroretentive layer was designed and prepared at a compression force that would ensure optimal floating lag time and total floating time values during *in vitro* buoyancy testing.

2.6 In vitro buoyancy

The floating behaviour of the bilayer tablets for the factorial experiment and optimised gastroretentive layer selection purposes was assessed in 500 mL fasted state simulated gastric fluid (FaSSGF) pH 1.6 in a USP II dissolution apparatus. The apparatus was set at a paddle rotation speed of 75 rpm and a temperature of 37 ± 0.5 °C. The *in vitro* buoyancy of the gastroretentive tablets was characterised by the floating lag time and total floating time parameters. Furthermore, the *in vitro* buoyancy of the optimised gastroretentive ethylcellulose-containing bilayer F_{HE6005} tablets was tested in HCl pH 1.0, phosphate buffer pH 3.0 and phosphate buffer 6.0 to assess any potential pH effects on the floating capabilities of the tablets.

2.7 Tablet specification, wet strength and density testing

The tablets prepared were assessed in terms of their hardness using a C50 Tablet Hardness tester (Engineering Systems (Nottm)®, Nottingham, United Kingdom). The hardness testing was performed as per USP <1217> [37]. The tablet dimensions (long axis diameter, short axis diameter, wall height, thickness) and hardness (kg units) were recorded, followed by calculation of the tablet tensile strength [38].

The friability testing of the tablets was conducted as per USP <1216> using a friability tester (Erweka®, Heusenstamm, Germany) [39]. As per USP, the friability should be less than 1.0 %.

The tablet weight variation testing was conducted as per USP <905> [40]. The tablet weight variation was considered to be within the acceptable range set in USP if the acceptance value was less than 15.

The dimensions of the hydrated bilayer tablets after *in vitro* buoyancy testing were measured using a Vernier calliper. The compression wet strength of the bilayer tablets was measured using a TA.XT Plus texture analyser (Stable Micro Systems®, Godalming, United Kingdom). A 50-kg load cell and a 2-cm-diameter flat-tipped cylinder probe were used for the test. Peak force was recorded in Newtons (N) at 85 % strain using the force-displacement curves.

The true density of the optimised bilayer tablets was determined using a helium pycnometer (AccuPyc® II 1340, Micromeritics Instrument Cooperation, Norcross, GA, USA) (n = 3 tablets). The instrument was calibrated using a standard stainless-steel sphere before measurements. The tablets were then accurately weighed and placed inside the chamber. The sample solid phase volume was calculated through the pressures observed upon filling the sample chamber and then discharging into another empty chamber. The temperature inside the chamber was maintained at 21.95 °C. The measurements were repeated for five cycles. The volume and the apparent (skeletal) density of the tablets were determined. Additionally, the porosity and pore size of the optimised bilayer tablets, as well as their skeletal and bulk density, was determined via mercury intrusion porosimetry (Autopore® IV 9500, Micromeritics Instrument Cooperation, Norcross, GA, USA) (n = 3 tablets). The tablets were accurately weighed before measurements. The pressure was varied from 0 to 70 psi. A 10 s equilibration time was set for each data point.

2.8 Scanning electron microscopy (SEM) analysis of the optimised gastroretentive layer of the tablets in different media

Images of the optimised gastroretentive layer were collected at different timepoints of the bilayer tablet *in vitro* buoyancy runs in HCl pH 1.0, phosphate buffer pH 3.0 and phosphate buffer pH 6.0 using scanning electron microscopy (SEM) (Philips® XL30 SEM, Netherlands). The timepoints selected were 12 and 22 hours in all media, 31 hours for the tablets in phosphate buffer pH 3.0 and phosphate buffer pH 6.0 and an additional 43.5-hour timepoint for the tablets in phosphate buffer pH 6.0. Before imaging, the tablet samples were carefully rinsed with water droplets to remove as much of the dissolution medium remnants as possible, were dried at 40 °C until constant weight and then sputtered with gold using an SC7640 Sputter Coater (Polaron®,

United Kingdom). Coated samples were then observed in the SEM at an accelerating voltage of 10 kV under vacuum conditions at ambient temperature.

2.9 Viscosity study

The viscosity study was conducted to obtain quantitative information on potential changes and/or differences in the tablet PEO content over time during the *in vitro* buoyancy test and between the different media which could, in turn, affect the floatation of the tablets in those media. Viscosity measurements were conducted using an Anton Paar® MCR 302 rheometer (Anton Paar, United Kingdom) set at a rotation speed of 2 s^{-1} and a temperature of 37 °C. The cone-and-plate setting was used. *In vitro* buoyancy runs of the optimised ethylcellulose-containing bilayer tablets were conducted in HCl pH 1.0, phosphate buffer pH 3.0 and phosphate buffer pH 6.0. Approximately 7 mL of sample were collected manually at certain timepoints (1, 4, 8, 12, 22 h in all media, 31 h in phosphate buffer pH 3.0 and phosphate buffer pH 6.0, and 43.5 h in phosphate buffer pH 6.0). Each time, after the viscosity measurement, the sample was transferred back to the dissolution vessel. The runs and viscosity measurements were conducted in triplicate in each medium.

2.10 In vitro release of MT-1207 from gastroretentive bilayer tablets

In vitro release testing was carried out using a USP II dissolution apparatus (Copley®, United Kingdom). The apparatus was set at a constant paddle rotation speed of 75 rpm. *In vitro* release testing was conducted in 500 mL 0.1 M acetate buffer pH 4.0 at 37 ± 0.5 °C. Stainless-steel sinkers were used to maintain the tablets at the bottom of the dissolution vessels to eliminate the effect of tablet floating (location of tablets within the vessels) on drug release [22, 41]. Sampling was carried out manually at predetermined time intervals (1, 2, 4, 6, 8, 12, 16 and 24 hours), followed by centrifugation of the samples (2300 g, 10 minutes). Then, each time, 1 mL from the supernatant was transferred into an HPLC vial and 20 µL were injected into the HPLC for analysis. The *in vitro* release study was conducted in triplicate for each formulation. Additionally, an *in vitro* release experiment was conducted at a paddle rotation speed of 200 rpm for the bilayer tablets containing the optimised ethylcellulose-containing drug layer (F_{HE6005}) and the bilayer A1 tablets.

2.11 Kinetics and mechanism of drug release

Based on the results obtained from *in vitro* release testing for the optimised formulations bilayer F_{HE6005} and bilayer A1, the release kinetics of MT-1207 from these formulations were studied. For this purpose, for each formulation, the data obtained from the *in vitro* drug release testing were plotted in various kinetic models.

These models were first-order (Equation 2) as log percentage of MT-1207 remaining undissolved vs time, zero-order (Equation 3) as the cumulative amount of drug released vs time and Higuchi's models (Equation 4) as cumulative percentage of MT-1207 released vs square root of time. Finally, the data were plotted using the Hixson-Crowell cube root law to evaluate the drug release with changes in the particle/tablet surface area and diameter (Equation 5) [42].

$$LogQ_1 = LogQ_0 - k_1 t/2.303 \quad (2)$$

where Q_1 is the amount of the drug in the tablet at time t, Q_0 is the total amount of the drug and k_1 is the first-order constant.

$$C = k_0 t \quad (3)$$

where C is the concentration of the drug at time t and k_0 is the zero-order constant.

$$Q = k_H t^{1/2} \quad (4)$$

where Q is the cumulative % of drug released at time t and k_H is a constant the value of which depends on the system design variables [42].

$$\sqrt[3]{Q_0} - \sqrt[3]{Q_t} = k_{HC} \times t \quad (5)$$

where Q_t is the amount of drug released in time t, Q_0 is the initial total amount of drug in the tablet and k_{HC} is the rate constant for the Hixson-Crowell equation which refers to the plot of the cube root of the drug remaining in the tablet vs time.

The mechanism of drug release from bilayer F_{HE6005} and bilayer A1 tablets was determined using the Korsmeyer-Peppas model [43]. The data from the *in vitro* release testing were plotted in the Korsmeyer-Peppas equation (Equation 6) as log cumulative percentage of drug released vs log time. The exponent n was calculated by measuring the slope of the straight line.

$$M_t/M_\infty = kt^n \quad (6)$$

where M_t is the amount of drug released at time t, M_∞ is the total amount of drug and k is a kinetic constant the value of which is characteristic of the drug/polymer system and n is an exponent that is used to characterise the mechanism of drug release [43]. For cylinder tablets, $n = 0.45$ corresponds to drug release controlled by Fickian diffusion, $0.45 < n < 1.00$ corresponds to anomalous (non-Fickian diffusion) which refers to a combination of Fickian diffusion and polymer relaxation mechanisms, while $n > 1.00$ defines super Case-II transport where drug release is controlled by polymer relaxation. For slabs, $n = 0.50$ corresponds to drug release controlled by Fickian diffusion, $0.50 < n < 1.00$ corresponds to non-Fickian diffusion [34, 44-46].

2.12 In vivo study

An *in vivo* pharmacokinetic study was conducted in Beagle dogs with a body weight of 10 ± 2 kg for MT-1207 30 mg gastroretentive bilayer F_{HE6005} and bilayer A1 tablets, and 10 mg immediate-release tablets. The study was conducted in China Pharmaceutical University (Nanjing, China). The study complied with the principles of Laboratory Animal Care and was approved by China Pharmaceutical University Animal Management and Ethics Committee (License number: SYXK (SU) 2021-0021; Month & Year of Approval: November 2021).

Four healthy male Beagle dogs were used. The dogs were provided by Nanjing Chai Men Biotechnology Co., Ltd. (Nanjing, China). Each time, the Beagle dogs were fasted overnight, and blank blood was collected shortly before the study. The Beagle dogs were then given free access to a meal and, after 30 minutes, one gastroretentive bilayer tablet or three immediate-release tablets were administered orally. 1.5 mL of venous blood were taken at 0.25 h, 0.5 h, 1 h, 2 h, 4 h, 6 h, 8 h, 12 h, 24 h and 32 h after administration and centrifuged in a tube with heparin. A one-week washout period was applied between the different dosing regimens. In all cases, each sample was placed in a test tube containing heparin, centrifuged and the plasma was separated and stored at -70 °C. The concentration of MT-1207 in plasma samples was determined using UPLC-MS/MS.

2.13 Bioanalytical method

The bioanalytical method that was used for the analysis of the samples collected during the pharmacokinetic study was the same as the one reported in our previous study [17]. In brief, MT-1207 in plasma samples collected during the pharmacokinetic study was determined by a validated UPLC-MS/MS method using

verapamil hydrochloride as an internal standard. Each time, 10 µL of plasma sample were pipetted in 1.5 mL Eppendorf® tube. 200 µL of verapamil hydrochloride 2 ng/mL in acetonitrile were added and vortex was carried out for 5 minutes. Centrifugation was then carried out at 15000 rpm for 5 minutes and 100 µL of supernatant were collected for UPLC-MS/MS analysis. The ion source was an electron spray ionisation (ESI) source. A positive ion scanning method was used for detection. The solvent gas (nitrogen) flow rate was 1000 L/h, the temperature of the solvent gas was 500 °C and the capillary voltage was 3.0 kV. The scanning method was Multiple Response Monitoring (MRM). The cone voltage was set at 40 V, while the collision energy was 20 eV. For quantitative analysis, the ion pairs used had m/z 393.26 → 274.04 (MT-1207) and m/z 455.25 → 156.06 (internal standard). The samples were applied to an ACQUITY Ultra Performance Liquid Chromatography system with Xevo TQ-XS Triple Quadrupole Mass Spectrometer with operating software MassLynx V4.2 (Waters Technology Limited Company). The column used was an ACQUITY UPLC BEH C18 liquid chromatography column (2.1 × 50 mm, 1.7 µm). The mobile phase consisted of 0.1 % formic acid in water (mobile phase A) and acetonitrile (mobile phase B). Verapamil hydrochloride was used as the internal standard for determination. The gradient elution was: 0 − 1.2 min: 20 – 45 % B, 1.2 − 1.5 min: 45-95 % B, 1.5 − 1.8 min: 95 % B, 1.8 − 2.5 min: 95 – 20 % B. The flow rate was set at 0.5 mL/min. The column temperature was set at 45 °C.

Quantification was carried out using a calibration curve. Working standard solutions with concentration values of 10, 20, 40, 100, 200, 400, 1000, 2000, 4000 ng/mL were prepared and used for the calibration curve. The concentrations of the quality control samples were 30, 320, 3200 ng/mL.

2.14 Data analysis

The *t*-test and one-way ANOVA statistical analyses of the data collected from the factorial experiment, the *in vitro* buoyancy study at media of different pH values, the viscosity analysis and the pharmacokinetic study were conducted using SigmaPlot® 13. A statistically significant difference was reflected by a *P* value less than 0.05. Except for the factorial experiment, in the cases where one-way ANOVA analysis gave a *p*-value less than 0.05, *post hoc* Bonferroni *t*-test was conducted for pairwise comparisons. The similarity or difference between the *in vitro* drug release profiles of the different formulations were assessed using the difference factor (f_1) and similarity factor (f_2), as per the FDA guidelines (Food and Drug Administration, 1997)[47]. Values of f_1 up to 15 (0 − 15) and f_2 greater than 50 (50 – 100) ensured sameness or equivalence of MT-1207 *in vitro* release profiles of different formulations.

Regarding the pharmacokinetic study, the half-life ($t_{1/2}$), clearance (CL), apparent volume of distribution (V_d), and mean residence time (MRT) of MT-1207 after administration of the tablets following the different dosing regimens were also calculated using WinNonlin® version 6.4. The peak plasma concentration of MT-1207 (C_{max}), the time to reach C_{max} (T_{max}) and the area under the concentration-time curve from 0 h to 24 h (AUC_{0-24h}) and to infinity ($AUC_{0-\infty}$) were calculated from the plasma concentration versus time profile using WinNonlin® version 6.4. Finally, the bioequivalence between the sustained-release and immediate-release tablets was investigated by calculating the ratio of AUC_{0-24h} values of the sustained-release tablet dosing regimen and the two different immediate-release tablet dosing regimens.

3 Results and Discussion

3.1 Flowability testing of gastroretentive and drug layer powder mixtures

Bulk/Tapped density testing was performed for the gastroretentive and drug layer powder mixtures to assess their flowability and compressibility. The assessment was based on the Carr index and Hausner ratio values calculated for each powder. A Carr index value between 5 % and 16 % is desirable to achieve both good flowability and compressibility [48].

All gastroretentive and drug layer powder mixtures gave Carr index and Hausner ratio values that indicated a passable to good flowability (Tables S1 and S2, Supplementary material) as per USP [49], along with a potentially good compressibility since the Carr index values were higher than 10 %. Therefore, the gastroretentive and drug layer powder mixtures were forwarded to tabletting.

3.2 Factorial experiment

The three different factors (amount of PEO, % of spray-dried lactose and compression force) potentially affecting the floating lag time and total floating time of the non-effervescent bilayer tablets were selected based on gastroretentive layer preliminary experiments. The two dependent variables (floating lag time and total floating time) were selected to assess the *in vitro* buoyancy behaviour of the gastroretentive tablets. The results of the factorial experiment for the different formulations can be seen in Table 3.

Table 1: Factorial experiment results for the different formulations, regarding their floating lag time and total floating time (n = 3 tablets). Results are expressed as mean ± standard deviation.

Formulation	Floating Lag Time (seconds)	Total Floating Time (Minutes)
1	0.9 ± 0.0	1783.7 ± 45.0
2	1.1 ± 0.2	1286.3 ± 41.7
3	0.0 ± 0.0	2050.0 ± 7.5
4	0.0 ± 0.0	1617.3 ± 84.2
5	9096.4 ± 67.6	603.0 ± 1.4
6	9069.5 ± 50.3	594.3 ± 41.5
7	12.8 ± 2.0	1508.3 ± 100.4
8	1.9 ± 0.2	1300.0 ± 11.5
9	0.7 ± 0.0	1719.7 ± 6.4
10	0.1 ± 0.1	1379.0 ± 27.5
11	3682.5 ± 90.7	867.0 ± 34.4
12	12579.4 ± 207.8	686.7 ± 49.4
13	216.7 ± 206.7	850.0 ± 130.1
14	267.6 ± 34.0	588.3 ± 29.9
15	0.0 ± 0.0	1120.3 ± 113.9
16	231.5 ± 1.7	922.7 ± 165.4
17	11950.9 ± 740.4	684.0 ± 177.4
18	22981.0 ± 10356.2	556.0 ± 110.9
19	1.8 ± 0.3	1479.5 ± 62.9
20	254.0 ± 64.1	1341.3 ± 187.9
21	0.0 ± 0.0	1207.0 ± 8.5
22	0.0 ± 0.0	1624.7 ± 28.6
23	0.0 ± 0.0	1613.0 ± 188.4
24	1585.8 ± 183.2	848.7 ± 55.4
25	1717.8 ± 190.4	988.0 ± 19.0
26	244.5 ± 3.3	892.0 ± 215.2
27	10676.0 ± 408.2	653.3 ± 37.1
Optimised	1.5 ± 0.8	1498.0 ± 75.4

The quadratic model was the best-fitting model for floating lag time ($p < 0.001$) and the relationship between the variable and the different factors was depicted in Equation 7. The compression force was the main factor significantly affecting the tablet floating lag time in both a linear and a non-linear positive manner (one-way ANOVA, $p < 0.05$ for the linear term, and $p < 0.01$ for the non-linear term). Increasing the compression force led to significantly longer floating lag time values. This could be attributed to an increase in the tablet density that was probably linked to a reduced tablet porosity in higher compression forces [24, 50]. Additionally, the ANOVA analysis demonstrated a potential interaction between the amount of PEO 8M and the compression force with a significant negative effect on the variable ($p < 0.05$). This trend was particularly noticed at a compression force of 7.5 kN, where larger amounts of PEO 8M contributed to shorter floating lag times. This

finding was in contrast to a previous report where increasing amounts of PEO resulted in longer floating lag times [24]. The material used in the present study had a lower bulk density of 0.3811 g/cm^3 and a larger mean particle size of 247.7 μm, compared to previous work [34]. This low density and relatively large particle size of PEO may have contributed towards lower tablet density and higher tablet porosity values with increasing amounts of the material in the gastroretentive layer, thus leading to shorter floating lag time values.

$$FLT = 21589.495 - (16.601 \times X_1) - (760.991 \times X_2) - (6665.087 \times X_3) + (1.687 \times X_1X_2) - (5.338 \times X_1X_3) - (187.326 \times X_2X_3) + (0.0647 \times X_1^2) + (186.544 \times X_2^2) + (725.058 \times X_3^2) \quad (7)$$

The model did not fit as well to the experimental data as might have been desired, since the R^2 value was 0.829 and the adjusted R^2 value was 0.738. Despite this, it was still able to demonstrate which factor and factor interaction had a significant impact on the floating lag time of the gastroretentive tablets. However, given the model fit, it was only used in an informative way to demonstrate which factors had an impact on floating lag time rather than to a predictive capacity. Based on the experimental data, a compression force value between 5 and 7.5 kN would be a good choice to ensure a minimal floating lag time which is the target for gastroretentive floating systems [24]. When considering the choice of the optimal compression force, though, apart from the floating lag time, the tablet hardness and the wet strength of the tablets after the *in vitro* buoyancy test also need to be considered. In Table S3, it can be seen that increasing compression force values had a statistically significant positive effect on the tablet hardness and wet strength, especially for high-weight tablets containing 320 mg and 480 mg PEO (one-way ANOVA, $p < 0.001$ and *post hoc* Bonferroni, $p < 0.05$ for pairwise comparisons regarding tablet hardness, one-way ANOVA, $p < 0.01$ and *post hoc* Bonferroni, $p < 0.05$ for pairwise comparisons regarding wet strength). The tablet hardness should be high enough to ensure the mechanical integrity of tablets during handling, while the wet strength of the tablets should be higher than 3.2 N which is the estimated gastric destructive force experienced in the stomachs of Beagle dogs [51]. Therefore, based on the experimental data, a compression force value close to 7.5 kN should be chosen as the optimal one since it would help ensure a short floating lag time of the tablets, alongside sufficient hardness and wet strength.

With regards to the total floating time, a quadratic model was again the best fit. The relationship between the variable and the factors and factor interactions can be seen in Equation 8.

$$TFT = -1014.457 + (8.808 \times X_1) + (107.952 \times X_2) + (280.496 \times X_3) + (0.0523 \times X_1X_2) - (0.394 \times X_1X_3) - (6.229 \times X_2X_3) - (0.00704 \times X_1^2) - (5.797 \times X_2^2) - (18.883 \times X_3^2) \quad (8)$$

The amount of PEO in the gastroretentive layer was the main factor affecting the total floating time of the tablets in both a linear and a non-linear trend (one-way ANOVA, $p < 0.001$ for the linear term and $p < 0.01$ for the non-linear term). The buoyancy of gastroretentive formulations has been reported to be strongly dependent on the swelling of the hydrocolloid particles that come in contact with the gastric fluids [52]. This is because, upon hydration, PEO swells, thus increasing tablet volume and reducing the density. At some point, the hydration volume is maximised, and polymer disentanglement and erosion become dominant, thus leading to volume reduction. Higher proportions of PEO can increase the tablet volume attained upon hydration and the time required for the hydration volume to become maximum, thus resulting in prolonged floating times [26]. Furthermore, increased amounts of PEO may result in the formation of a stronger gel [24] which could slow the gel erosion process and can be a limiting factor for the magnitude and maintenance of the hydrated tablet size [34]. The tablet size is linked to hydrated tablet density and, thus, floatability. Hence, these factors may have contributed to the increasing tablet total floating time values recorded with increasing amounts of PEO in the gastroretentive layer.

Apart from the amount of PEO, the compression force was also a significant factor affecting the total floating time in a non-linear manner (one-way ANOVA, $p < 0.05$). Higher compression forces can lead to a reduced internal porosity of the tablets. In hydrophilic matrix tablets, it has been demonstrated that the reduced tablet porosity could lead to a reduced water transport rate through the matrix during contact with aqueous media [53]. This could result in longer times to achieve maximum hydration volume, as well as lower levels of maximum hydration volume, compared to tablets compressed using lower forces [26]. Furthermore, visual observation of the tablet floating behaviour upon application of vertical pressure showed that tablets compressed using lower compression forces had a higher tendency to re-surface on the medium. This trend was also noted in a study by Goole et al. [54] and may have contributed towards a more efficient and robust floatation leading to longer total floating times in lower compression force values.

A significant negative effect on the total floating time was the interaction between the amount of PEO and the compression force (one-way ANOVA, $p < 0.001$). As discussed, these two factors had opposite effects on the dependent variable. The prevailing negative effect of the combined factors on the total floating time was because the negative effect of the compression force on the variable was dominant for higher tablet weights and,

therefore, higher amounts of PEO, especially for the tablets that contained 320 mg and 480 mg PEO (one-way ANOVA, $p < 0.001$, and *post hoc* Bonferroni $p < 0.01$ for pairwise comparisons). Clear differences were recorded in the total floating time for the same tablet formulas under different compression force values (Table 3). Finally, the percentage of spray-dried lactose in the gastroretentive layer had a minor positive effect on the total floating time of the tablets, but it was not statistically significant (one-way ANOVA, $p > 0.05$).

The total floating time quadratic model fitted the experimental data well, indicated by an R^2 value of 0.923 and an adjusted R^2 value of 0.883. However, it was realised that, if the non-significant linear term of compression force was eliminated from the equation, the model fit to the data would improve. Therefore, Equation 9 was generated with an R^2 value of 0.921 and an adjusted R^2 value of 0.887.

$$TFT = 41.919 + (8.470 \times X_1) + (100.213 \times X_2) + (0.0523 \times X_1X_2) - (0.348 \times X_1X_3) - (5.197 \times X_2X_3) - (0.00704 \times X_1^2) - (5.797 \times X_2^2) - (1.548 \times X_3^2) \quad (9)$$

Kollidon® SR was a major component of the gastroretentive layer. The preliminary gastroretentive layer study demonstrated that it did not have a significant effect on the floating lag time and total floating time of tablets. However, high percentages of Kollidon® SR did lead to higher tensile strength and higher wet strength values of the tablets, measured after completion of the *in vitro* buoyancy tests. Based on these results, the percentage of Kollidon® SR selected for all the factorial experiment runs (34.0 %) was deemed crucial in ensuring an adequately high wet strength of the tablets, apart from a sufficiently high compression force.

Based on Equation 9, as well as floating lag time, tablet hardness and tablet wet strength considerations, a candidate optimised gastroretentive layer was designed and a compression force of 7 kN was selected for the tablet preparation. The gastroretentive tablets were prepared using a simple direct compression process. The composition of the optimised layer is in Table 5. The bilayer tablets containing this layer were characterised in terms of their *in vitro* buoyancy properties. As can be seen in Table 3, the tablets demonstrated a negligible floating lag time and a total floating time that was longer than 24 hours and close to the theoretical value calculated via Equation 9. This further confirmed the suitability of the model to predict the total floating time values of formulation combinations. Also, the hardness and tensile strength values of the tablets were sufficiently high to ensure their mechanical integrity (Table S4, Supplementary material), while their wet strength value of 8.7 ± 1.6 N after the *in vitro* buoyancy test could contribute to their integrity *in vivo*. Finally, in terms of the swollen tablet dimensions, after sinking, the tablets had a long axis diameter of 25.8 ± 0.8 mm, a

short axis diameter of 13.7 ± 0.6 mm and a thickness of 13.2 ± 1.1 mm (n = 3 tablets). These dimensions were larger on average than the estimated average pyloric sphincter diameter of 12.8 mm [55, 56]. This could further contribute towards extended gastric retention of the tablets, alongside their prolonged buoyancy. Therefore, this gastroretentive layer was selected as the optimal.

The *in vitro* buoyancy of bilayer A1 tablets in FaSSGF pH 1.6 was characterised since they were also considered promising as a once-a-day oral formulation for MT-1207. Their floating lag time was 1.8 ± 0.5 seconds and their total floating time was 1599.7 ± 130.0 minutes. Additionally, after the *in vitro* buoyancy run, their wet strength was sufficiently high (6.9 ± 1.4 N), and, in terms of their swollen dimensions, their long axis diameter was 26.1 ± 0.7 mm, their short axis diameter was 13.5 ± 0.5 mm and their thickness was 12.9 ± 0.5 mm (n = 3 tablets). The negligible floating lag time and total floating time longer than 24 hours confirmed the promising gastroretentive application of the bilayer A1 tablets. Additionally, there was no statistically significant difference in the floating lag time and total floating time values between the bilayer A1, F_{HE6005} and F_{HE7035} (floating lag time of 1.0 ± 0.9 s, total floating time of 1557.3 ± 126.9 minutes) tablets (one-way ANOVA, $p > 0.05$). These findings indicate that the *in vitro* buoyancy behaviour of the bilayer tablets was mainly dependent on the gastroretentive layer with no significant effect from the drug layer.

The factorial experiment contributed to the development of a novel non-effervescent gastroretentive layer that was easy to prepare, provided sufficiently high swollen tablet dimensions over 24 hours, and ensured immediate tablet buoyancy that lasted over 24 hours in FaSSGF pH 1.6 and was independent of the drug layer.

3.3 Post-compression characterisation of tablets

The hardness values of the prepared tablet formulations containing the different drug layers can be seen in Table S4 (Supplementary material). There was no trend in the tensile strength between different formulations. Therefore, the content of ethylcellulose did not seem to have a significant effect on tablet tensile strength, despite the fact that it has binder properties [57]. The reason for this could be that in many of these formulations PVP K30 was also included as a binder which could have affected the tensile strength of the tablets. All formulations demonstrated sufficiently high tablet hardness and tensile strength values to ensure their mechanical integrity during transportation or other stresses.

The thickness of the tablets containing the optimised gastroretentive layer ranged between 7.3 and 7.7 mm and the diameter of the semicircle on each side of the oval biconvex tablets was 1 mm. Furthermore, the friability of all bilayer tablets was less than 1.0 % (Table S4, Supplementary material) which further indicated the ability of the tablets to withstand the expected mechanical shocks during handling and transportation, as per

USP [39]. Finally, all tablet batches gave acceptable mean weight values and acceptance values below 15.0, with regards to tablet weight variation (Table S5, Supplementary material). Therefore, for all formulations, the tablets were considered uniform in weight, as per USP [40].

3.4 Tablet density and porosity characterisation

Information on the density and porosity of the optimised F_{HE6005} bilayer tablets was provided through helium pycnometry and mercury intrusion porosimetry analyses. The tablet volume was 2.0119 ± 0.0033 cm^3. The total mercury intrusion volume was 0.2381 ± 0.0050 mL/g, the volume pore diameter was 1.40 ± 0.14 μm and the tablet porosity was 23.7 ± 0.5 %. As per a previous report, this level of tablet porosity can be considered to be relatively high with tablet porosity values higher than 20 % linked to low tensile strength [24]. However, this was not the case in the present study since the optimised bilayer F_{HE6005} tablets were characterised to have sufficiently high hardness and tensile strength values (Table S3, Supplementary material).

Both helium pycnometry and mercury intrusion porosimetry methods provided tablet skeletal density information. The values reported using the different methods were consistent. The tablet skeletal density was estimated to be 1.2875 ± 0.0015 g/cm^3 using helium pycnometry and 1.3051 ± 0.0497 g/cm^3 using mercury intrusion porosimetry. However, the tablet bulk density determined using mercury intrusion porosimetry was 0.9957 ± 0.0326 g/cm^3. The bulk density calculation takes the air-filled pore spaces within the tablets into account, in contrast to the skeletal density measurements. Therefore, since the tablet density on average was lower than 1.004 g/cm^3, it was inferred that this was most likely the reason for the nearly immediate floatation of the optimised bilayer tablets in aqueous media.

3.5 In vitro buoyancy in media of different pH values

The buoyancy capability of floating gastroretentive tablets is critical in ensuring robust gastric retention *in vivo*. Non-effervescent gastroretentive formulation applications benefit from the fact that their floating lag time is not affected by the gastric pH, thereby enabling their suitability for patients with achlorhydria [21]. Furthermore, other factors, such as sugar and salt stomach contents during fed state, have been reported to affect the swelling properties of polymers such as HPMC, and, thus, the floatation of gastroretentive formulations [21]. However, to the best of our knowledge, no study has investigated the effect of pH on the total floating time of non-effervescent gastroretentive formulations.

In the present study, the *in vitro* buoyancy of the optimised gastroretentive bilayer F_{HE6005} tablets was assessed in media of different pH values, namely, HCl pH 1.0, phosphate buffer pH 3.0, and phosphate buffer

pH 6.0. The media were selected to cover a pH range 1.0 – 6.0 where human gastric pH usually falls in during fasted and fed states, although sometimes it can assume higher values [58]. The floating lag time and total floating time values recorded in each of the different media can be seen in Table 4.

Table 4: *In vitro* buoyancy testing results of the optimised gastroretentive bilayer F_{HE6005} tablets in HCl pH 1.0, phosphate buffer pH 3.0 and phosphate buffer pH 6.0. Results are presented as mean ± standard deviation.

Medium	Floating Lag Time (Seconds)	Total Floating Time (Minutes)
HCl pH 1.0	1.00 ± 0.88	1335.7 ± 149.3
Phosphate buffer pH 3.0	1.70 ± 0.44	1854.0 ± 152.0
Phosphate buffer pH 6.0	1.72 ± 0.15	2232.3 ± 88.2

There was no statistically significant difference between the tablet floating lag time values recorded in the different media (one-way ANOVA, $p > 0.05$). This was expected since the immediate buoyancy of the tablets was most likely achieved due to their bulk density being lower than 1.004 g/cm^3, as demonstrated during the helium pycnometry study. However, there was a statistically significant difference in the total floating time values of the tablets in the different media (one-way ANOVA, $p < 0.001$). The pairwise comparisons conducted as part of the *post hoc* Bonferroni *t*-test demonstrated significant differences between the tablet total floating time values recorded in HCl pH 1.0 and phosphate buffer pH 3.0 ($p < 0.01$), HCl pH 1.0 and phosphate buffer pH 6.0 ($p < 0.001$), and phosphate buffer pH 3.0 and phosphate buffer pH 6.0 ($p < 0.01$).

To the best of our knowledge, a significant pH effect on the total floating time of non-effervescent gastroretentive formulations has not been reported in the literature. Apart from MT-1207, the rest of the tablet components were non-ionic and, thus, their dissolution and/or swelling behaviour could not have been affected by the medium pH. Therefore, the reason for the recorded differences could lie with other physicochemical attributes of the excipients.

From the factorial experiment for the gastroretentive layer, it was concluded that the major factors affecting the total floating time were the amount of PEO, the compression force and the interaction between these two factors. Previous studies have demonstrated that PEO most likely undergoes degradation in strongly acidic pH. McGary first reported relevant findings where it was concluded that PEO degradation was accelerated in aqueous solutions in the presence of strong acids, as determined by a reduction in solution viscosity [59]. The reason for this was probably not only acid hydrolysis of the polymer taking place, but also an additional free-radical mechanism. It was believed that hydroperoxides that are present in the polymer could

undergo ionic rearrangement within the polymer structure that may result in chain scission [59]. Additionally, the results of a study conducted by Crowley et al. showed that small quantities of ascorbic acid could significantly reduce the molecular weight of PEO through an acid catalysed chain scission reaction [60]. Finally, Yang et al. reported that the acid effect on PEO could be enhanced at higher temperatures. FTIR spectra of Pluronic P123 aqueous solutions in the presence of 2 M HCl showed two additional peaks at temperatures equal to or higher than 36 °C, compared to the spectra of the same solutions analysed at lower temperatures. The presence of these peaks could be attributed to the C-O-C stretch mode of small PEO segments derived from ether bond cleavage. Therefore, it seemed that temperature values equal to or higher than 36 °C could contribute to the PEO acid-induced degradation [61].

PEO was a major component of the gastroretentive layer of the bilayer tablets and a significant factor affecting their floatation. Based on the abovementioned information from previous studies, it was hypothesized that the statistically significant differences between the tablet total floating time values recorded in the different media could be attributed to differences in the rate and extent of PEO degradation in the media. To investigate this hypothesis, an SEM analysis of the gastroretentive layer of the tablets was performed at selected timepoints during *in vitro* buoyancy testing to visualise the layer and how it may have changed over time, alongside a viscosity analysis of the solutions at different timepoints to provide quantitative information on their PEO content at certain timepoints.

Figure 1 illustrates SEM images of the optimised gastroretentive layer of the optimised tablets in their dry state. The surface of the gastroretentive layer appeared to be quite porous with most of it covered by irregular, porous particles, proposed to be PEO, that were well mixed with spherical Kollidon® SR particles.

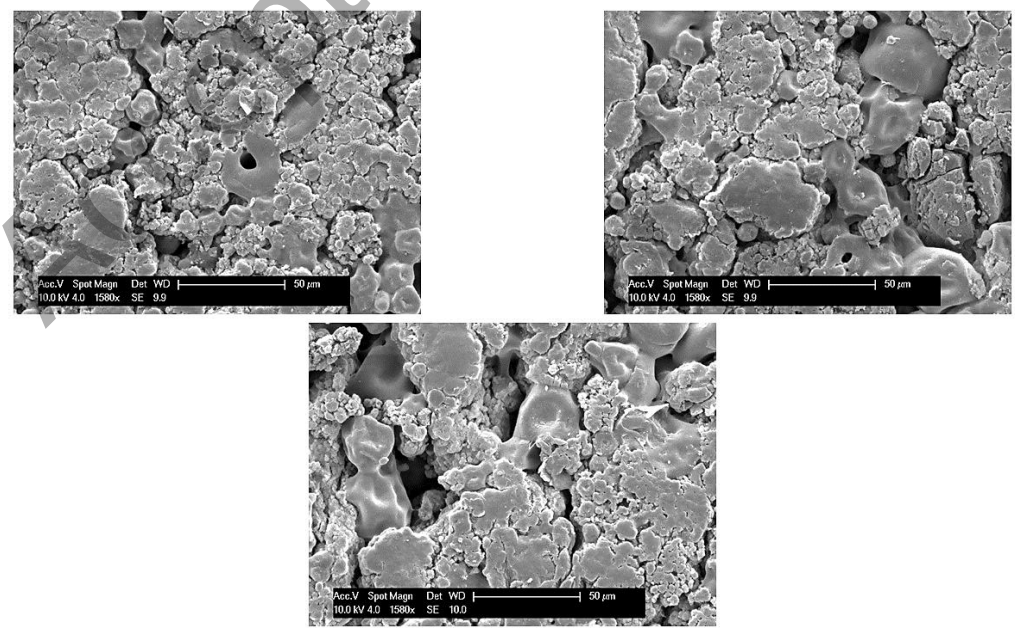


Figure 1: SEM images of the optimised gastroretentive layer of the dry bilayer tablets.

Figure 2 illustrates SEM images of the gastroretentive layer of the bilayer tablets during *in vitro* buoyancy in the different media at the 12-hour, 22-hour and 31-hour timepoints.

After 12 hours of *in vitro* buoyancy testing, the surface of the gastroretentive layer appeared fibrous in all media. These fibres probably contained amorphous PEO polymer that occurred from drying of the swollen PEO gel that had formed during the *in vitro* buoyancy test. The spherical particles present in the different images corresponded to Kollidon® SR particles. The small, irregular particles probably corresponded to PEO that crystallised during the drying process [62]. Based on the above, it could be assumed that at the 12-hour timepoint of the *in vitro* buoyancy test there was a strong presence of PEO in the gastroretentive layer of the tablets in the different media.

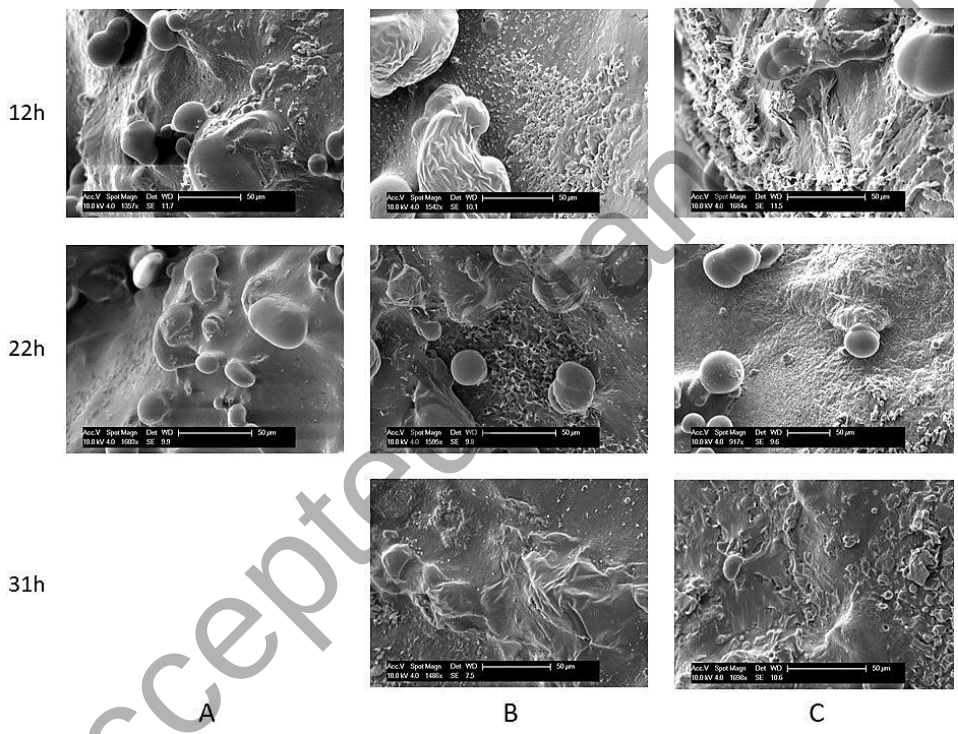


Figure 2: SEM images of the optimised gastroretentive layer of the bilayer tablets after 12, 22 and 31 hours of *in vitro* buoyancy testing in (A) HCl pH 1.0, (B) phosphate buffer pH 3.0, and (C) phosphate buffer pH 6.0. No SEM images were collected at the 31-hour timepoint in HCl pH 1.0 since tablet sinking in that medium occurred after 22 hours of *in vitro* buoyancy testing.

The 22-hour timepoint was the average sinking time of the tablets in HCl pH 1.0. It seemed that the presence of fibres in the gastroretentive layer of the tablets was much weaker after 22 hours of *in vitro* buoyancy

test in HCl pH 1.0, compared to the 12-hour timepoint in the same medium and the 22-hour timepoint in the other media. As seen in Figures 2B and 2C for the 22-hour timepoint, the surface of the tablet gastroretentive layer was characterised by a strong presence of fibres and small irregular particles that were believed to correspond to amorphous and crystallised PEO, respectively. The presence of fibres was more evident in the gastroretentive layer of the tablets tested in phosphate buffer pH 6.0. Therefore, it could be assumed that the presence of PEO in the gastroretentive layers of the tablets tested in phosphate buffer pH 3.0 and phosphate buffer pH 6.0 was still strong at the 22-hour timepoint of the *in vitro* buoyancy experiment.

The 31-hour timepoint was the average sinking time of the tablets in phosphate buffer pH 3.0. As seen in Figure 2B for that timepoint, the presence of PEO in the form of fibres and irregular particles was still noticeable in the gastroretentive layer of the tablets after 31 hours of *in vitro* buoyancy testing in phosphate buffer pH 3.0. However, the presence of PEO was much weaker, compared to the 22-hour timepoint of the tablet *in vitro* buoyancy test in phosphate buffer pH 3.0 and the 31-hour timepoint of the tablet *in vitro* buoyancy test in phosphate buffer pH 6.0 (Figure 2C).

Figure 3 shows SEM images of the gastroretentive layer of the bilayer tablets during *in vitro* buoyancy in phosphate buffer pH 6.0 at the 43.5-hour timepoint which was the average sinking time of the tablets in the medium. The presence of PEO in the form of fibres was still clear, even after 43.5 hours of *in vitro* buoyancy testing. However, the surface of the layer had become quite porous which indicated that erosion may have taken place during the *in vitro* buoyancy testing between the 31- and 43.5-hour timepoints.

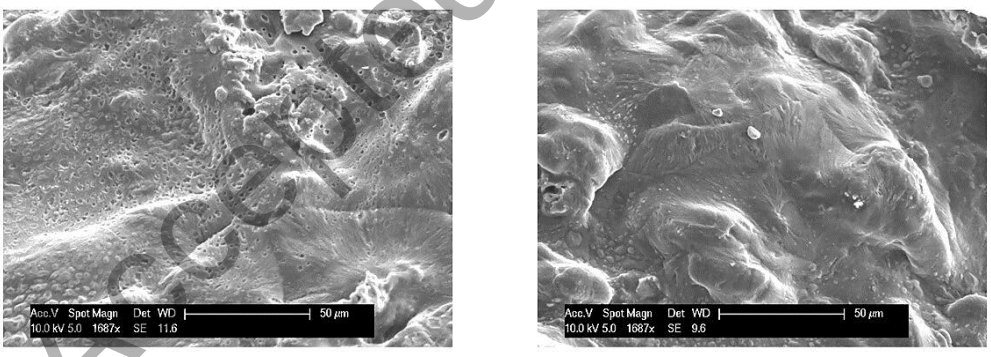


Figure 2: SEM images of the optimised gastroretentive layer of the bilayer tablets after 43.5 hours of *in vitro* buoyancy testing in phosphate buffer pH 6.0.

The SEM imaging indicated potential differences in the presence of PEO in the gastroretentive layer of the tablets between the different media in which *in vitro* buoyancy testing was performed, as well as between different timepoints of the test within the same medium. Therefore, the statistically significant differences in the

tablet total floating time values between the different media could be linked to differences in the rate and extent of PEO degradation in the different aqueous solutions. However, the SEM imaging only provided qualitative information and data for testing this hypothesis. To obtain more solid information on the trueness of the hypothesis, the acquisition of quantitative data was deemed crucial.

Viscosity analysis of the different media in different timepoints during the tablet *in vitro* buoyancy testing was performed to obtain quantitative information on potential changes and/or differences in the PEO content over time during the buoyancy test and between the different media, respectively (Figure 4).

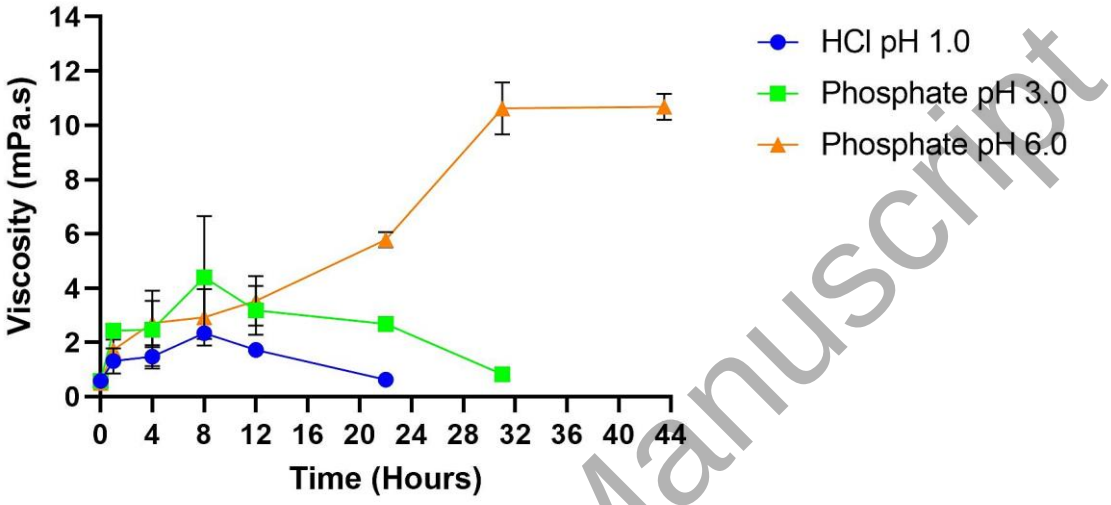


Figure 3: Viscosity of HCl pH 1.0, phosphate buffer pH 3.0 and phosphate buffer pH 6.0 media during *in vitro* buoyancy testing of the optimised gastroretentive bilayer F_{HE6005} tablets (n = 3). Results are presented as mean ± standard deviation.

Up to 12 hours, there was no statistically significant difference between different media in terms of the recorded viscosity values (one-way ANOVA, $p > 0.05$). This was in alignment with the SEM images at the same timepoint (Figure 2) where the tablets seemed to have a strong presence of PEO in the gastroretentive layer in the different media.

At the 22-hour timepoint, which was the approximate average sinking time of the bilayer tablets in HCl pH 1.0, statistically significant differences were recorded in the viscosity values between all different media (one-way ANOVA, $p < 0.001$, *post hoc* Bonferroni, $p < 0.001$). These findings, too, were aligned with the SEM images for the same timepoint (Figure 2) which indicated differences in the PEO content of the gastroretentive layer of the tablets in the different media. The SEM images for tablets in HCl pH 1.0 at the 22-hour timepoint showed the weak presence of PEO in the gastroretentive layer at this average sinking time of the tablets in the

medium. Additionally, at the 31-hour timepoint, which was the approximate average sinking time of the bilayer tablets in phosphate buffer pH 3.0, there was a statistically significant difference between the viscosity values recorded in phosphate buffer pH 3.0 and phosphate buffer pH 6.0 (*t*-test, $p < 0.01$). These findings were again in alignment with the respective SEM images (Figure 2) which indicated potential differences in the PEO content of the gastroretentive layer of the tablets in the different media. Therefore, it can be asserted that the differences in the *in vitro* buoyancy behaviour of the bilayer tablets in the different media could be attributed to differences in the rate and extent of PEO degradation in the different media depending on their acidity. This potential acid-induced degradation could further be conferred by the temperature, since 37 °C is considered high enough to induce chain scission within the polymer, based on previous, abovementioned findings [61].

A final viscosity measurement was conducted in phosphate buffer pH 6.0 after 43.5 hours of *in vitro* buoyancy testing which was the approximate average sinking time of the tablets in that medium. There was no statistically significant difference between the viscosity values recorded at 31 hours and 43.5 hours (*t*-test, $p > 0.05$). This indicated that polymer degradation in this nearly neutral medium was probably very slow, and that polymer swelling had probably peaked by 31 hours, thus resulting in similar medium viscosity values at the two timepoints. This result was in agreement with the SEM images of the gastroretentive layer collected at the same timepoint (Figure 3) since the presence of PEO in the layer had been estimated to be still significant. Therefore, in this case, the tablets probably sank due to their extensive hydration which has also been reported to affect the duration of the buoyancy of floating systems [63].

Our findings demonstrate the potential of pH effect on the buoyancy behaviour of non-effervescent gastroretentive formulations. These results further stress the importance of taking the physicochemical and stability properties of formulation components into account during the development of gastroretentive formulations. The results of the present study demonstrate that PEO-based floating systems should potentially be administered after a meal, not only because of the lower gastric emptying rates and the disruption of the migrating myoelectric complex (MMC) during the stomach fed state, but also because of the elevated gastric pH during that state. The weakly acidic to neutral pH conditions of the human stomach during the fed state could potentially promote the buoyancy of PEO-based formulations, due to a much slower degradation of the polymer, compared to the strongly acidic conditions prevailing during the fasted state. However, potential effects of other factors, such as sugar and/or salt stomach contents, on the buoyancy of PEO-based formulations should also be investigated.

3.6 In vitro release study

In our previous study, 0.1 M phosphate buffer pH 6.8 with 0.2% w/v SDS was used as the dissolution medium for *in vitro* release testing [17]. Based on that study results, single-layer A1 tablets containing HPMC K15M as the release retardant were selected as the optimised formulation. Even though a 24-hour release of MT-1207 from the tablets was demonstrated *in vitro*, the results in Beagle dogs indicated a much more rapid release of the active pharmaceutical ingredient (API) *in vivo* [17]. Since the solubility of MT-1207 in the medium was 0.4 mg/mL, it was considered a potential limiting factor for drug release, although sink conditions were theoretically established, as per the USP guidelines [64]. Therefore, the phosphate buffer dissolution medium was replaced by 0.1 M acetate buffer pH 4.0 where MT-1207 had a solubility value of 6.5 mg/mL.

The A1 tablets were tested for their *in vitro* drug release in the new medium. As seen in Figure 5, MT-1207 was completely released from the tablets within 12 hours. This finding contrasts with the 24-hour release profile that was recorded in 0.1 M phosphate buffer pH 6.8 with 0.2% w/v SDS. The release profile in the acetate buffer was closer to the *in vivo* data. Furthermore, the inability of HPMC K15M to retard the release of MT-1207 from the single-layer tablets over 24 hours in acetate pH 4.0 was demonstrated.

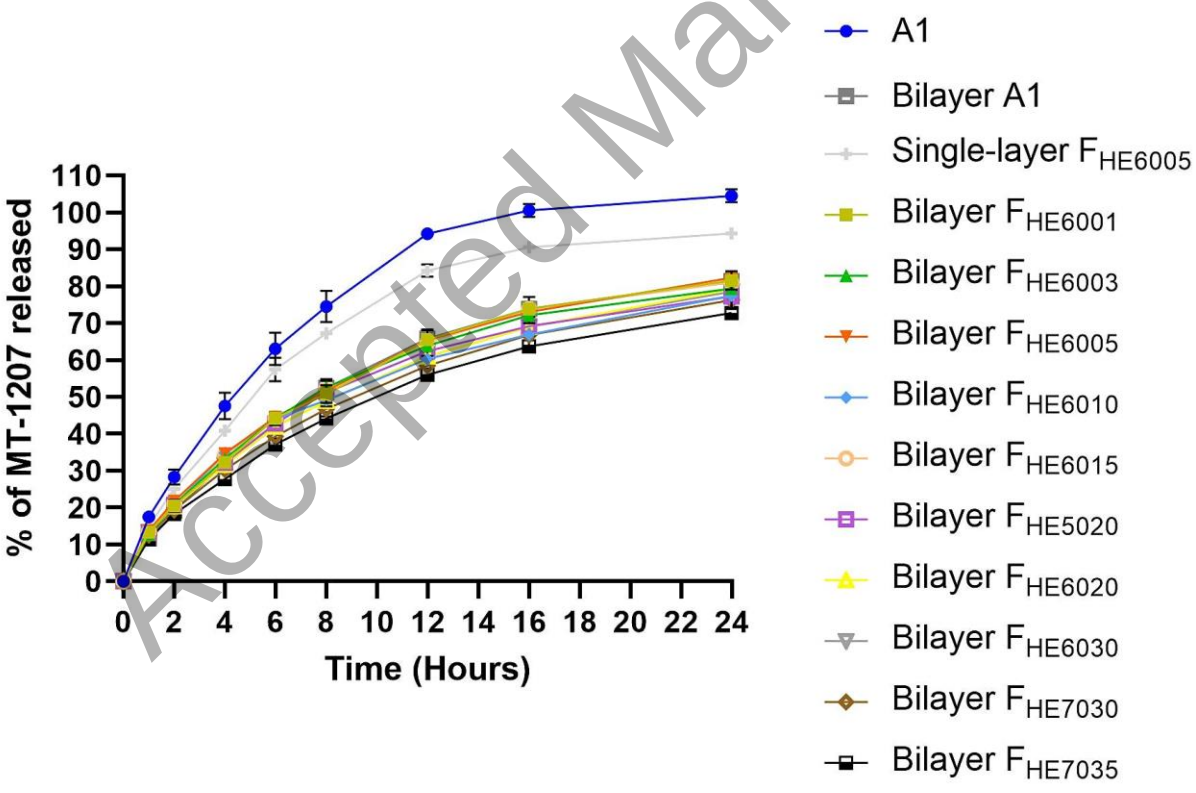


Figure 4: *In vitro* drug release profiles of A1, single-layer F_{HE6005} and the bilayer tablet formulations in 0.1 M acetate pH 4.0 at 75 rpm (n = 3 tablets). Results are presented as mean ± standard deviation.

As part of the once-a-day formulation optimisation, bilayer tablets were prepared and their *in vitro* drug release was assessed. Ethylcellulose 10 cps was incorporated as an additional release retardant in the drug layer, due to its hydrophobic polymer properties. HPMC K15M and ethylcellulose are intended to be able to retard the release of drugs from a formulation in different ways. HPMC K15M swells upon contact with aqueous media and forms a thick gel layer that is able to slow drug diffusion through the polymer chain network [65, 66]. Ethylcellulose can be effective in retarding drug release through a reduction in the penetration of solvent molecules into the matrix, due to the hydrophobicity of the polymer. This, in turn, could lead to reduced drug solubilisation and diffusion through the polymer matrix [67, 68].

The release profiles of MT-1207 from the different bilayer tablet formulations can be seen in Figure 5. The concentration of ethylcellulose varied from 0.5 to 19% w/w in the different formulations. Furthermore, bilayer A1 tablets were prepared and tested for their *in vitro* drug release. Incorporation of the gastroretentive layer into the formulation led to a statistically significant difference in the *in vitro* drug release profiles between the bilayer and single-layer A1 formulations ($f_1 = 40$, $f_2 = 36$) which indicated the ability of the bilayer tablets to retard the release of MT-1207 more effectively. The bilayer A1 tablets were able to provide a 24-hour sustained release of MT-1207 *in vitro* with more than 80 % of the API released at 24-hours. Therefore, this formulation was deemed promising for the once-a-day oral delivery of MT-1207. Additionally, there was no statistically significant difference in the *in vitro* release profiles between the bilayer A1 tablets and the bilayer tablets with the highest content of ethylcellulose (F_{HE7035}) ($f_1 = 13$, $f_2 = 59$). Therefore, the factor which significantly affected the release of MT-1207 from the tablets was not ethylcellulose, but the presence of the gastroretentive layer. This significant effect could be attributed to a much lower surface area of the drug layer that was exposed to the dissolution medium during the *in vitro* release testing. This was in contrast to previous studies which have reported the effectiveness of ethylcellulose as a release retardant, due to its very high hydrophobicity, compared to other hydrophobic polymers that have also been reported as release retardants. The relatively small particle size of ethylcellulose 10 cps (mean particle size of 100.6 μm; ethylcellulose of higher viscosities have particle sizes of 300 μm or more) could also have contributed to retardation of drug release [69, 70]. Other reports have also attributed this capability of ethylcellulose to percolation theory, where a small particle size of polymeric release retardant(s), relative to drug particle size, contributed to lower polymer percolation thresholds, thus potentially resulting in slowing of drug release at lower polymer concentrations [71, 72]. Finally, variations of

the HPMC K15M content in the drug layer between different formulations did not seem to induce any statistically significant effects on the *in vitro* release profile of MT-1207 (f_1 = 10, f_2 = 68).

Even though ethylcellulose did not seem to have a significant effect on *in vitro* drug release, it could potentially affect the release of MT-1207 *in vivo*. Despite the insignificant differences in the *in vitro* release profiles between the different ethylcellulose-containing formulations, F_{HE6015} and F_{HE6005} were the only formulations that provided a complete release of MT-1207, i.e., higher than 80 % on average, as per the European Medicines Agency (EMA) guidelines [73]. F_{HE6005} was the only formulation where all three tablets released more than 80 % of MT-1207 over 24 hours. Based on this additional consideration, F_{HE6005} was selected as the optimised ethylcellulose-containing drug layer to be formulated into the gastroretentive bilayer tablets.

An *in vitro* release experiment using a paddle rotation speed of 200 rpm was conducted for bilayer F_{HE6005} and bilayer A1 tablets. This rotation speed has been reported to better simulate the *in vivo* conditions of fasted Beagle dogs [74]. The results for both formulations demonstrated a sustained release of MT-1207 from the tablets over 24 hours even under those high shear conditions (Figure 6). For both formulations, the *in vitro* drug release profiles at 200 rpm were statistically similar to respective data recorded at 75 rpm (f_1 = 2 and f_2 = 89 for F_{HE6005} bilayer tablets, f_1 = 6 and f_2 = 76 for bilayer A1 tablets). Since the drug release was unaffected by conditions mimicking the Beagle dog destructive forces, it was probably largely independent of matrix erosion. Therefore, bilayer F_{HE6005} and bilayer A1 were selected as the optimised formulations. The details of the final optimised bilayer tablet formulations can be seen in Table 5.

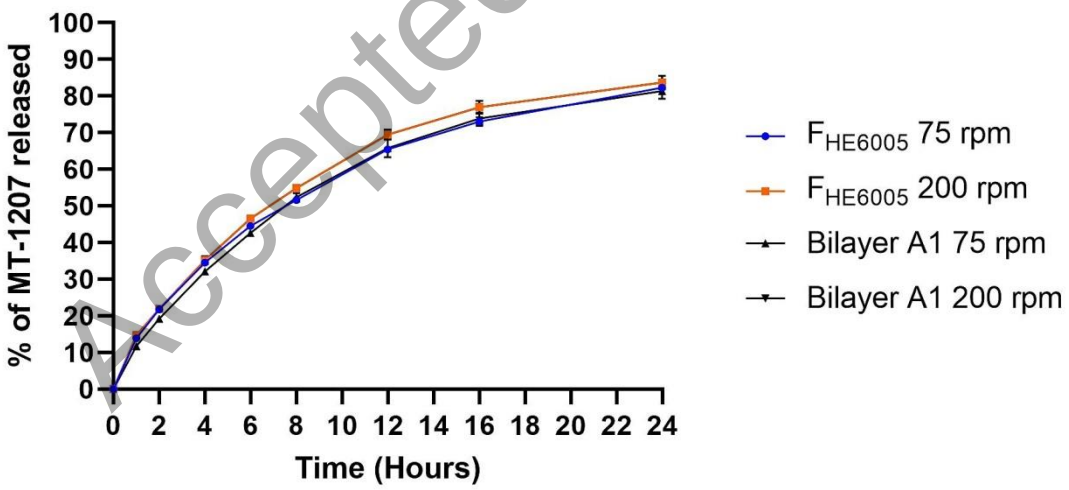


Figure 5: *In vitro* release profiles of MT-1207 from bilayer F_{HE6005} and bilayer A1 tablets at paddle rotation speeds of 75 rpm and 200 rpm (n = 3 tablets). Results are presented as mean ± standard deviation.

Table 2: The optimised bilayer tablet formulation (F_{HE6005} and A1) details.

Gastroretentive layer		
Components	Amount (mg)	
PEO 8M	380	
Spray-dried lactose	47	
Kollidon® SR	228	
HPMC K100M	16.2	
Colloidal silicon dioxide	1.5	
Magnesium stearate	1.5	
Total layer weight (mg)	**674.2**	
Drug layer	**F_{HE6005}**	**A1**
Components	Amount (mg)	Amount (mg)
MT-1207 Hydrochloride	30	30
Alpha-D-lactose monohydrate	65	99
Polyvinylpyrrolidone K30	20	20
HPMC K15M	60	70
Ethylcellulose 10 cps	5	-
Iron oxide	1	1
Colloidal silicon dioxide	6	6
Magnesium stearate	0.5	0.5
Total layer weight	**187.5**	**226.5**
Total tablet weight	**861.7**	**900.7**

3.7 Kinetics and mechanism of drug release from F_{HE6005} and bilayer A1 tablets

The release profiles of the bilayer F_{HE6005} and bilayer A1 tablets were plotted against different models to elucidate the kinetics of drug release from each formulation, while the Korsmeyer-Peppas model was used to obtain information on the potential mechanism of drug release. The release data that were plotted against the different models were those up to 60 % of their respective release curves [44, 75].

The release data of formulations bilayer F_{HE6005} and bilayer A1 were plotted in the first-order, zero-order, Higuchi, and Hixson-Crowell models. The highest R^2 value recorded was used to determine the best fit for each of the release profiles. The drug release from bilayer F_{HE6005} tablets followed a combination of first-order (R^2 = 0.9989) and Higuchi (R^2 = 0.9963) kinetics. When the data were plotted against the Korsmeyer-Peppas model, the *n* value (calculated from the slope of the curve) was 0.66 (Table 6) which, despite the complex geometry of the drug layer and the tablets, most likely indicated a non-Fickian diffusion mechanism of release of MT-1207 from F_{HE6005}. Therefore, it can be proposed that the kinetics of drug release from bilayer F_{HE6005} is best described as the release of MT-1207 from an intact matrix, the rate of which declined over time due to a reduction in the drug concentration within the tablet matrix. The mechanism of the release of MT-1207 from bilayer F_{HE6005} tablets hence resembles a combination of drug diffusion and polymer matrix erosion. The same assumptions on the kinetics and mechanism of drug release were also followed for the kinetic analysis of

drug release from bilayer A1 tablets (Table 7). However, there was a statistically significant difference in the *n* values recorded for the different formulations when the data were plotted against the Korsmeyer-Peppas model (*t*-test, $p < 0.01$). Therefore, matrix erosion was more significant for the release of MT-1207 from bilayer A1 tablets than for its release from bilayer F_{HE6005} ones. The small percentage of ethylcellulose in F_{HE6005} may have contributed towards a more intact drug layer during dissolution, an attribute that was desirable since it could potentially further ensure the integrity of the drug layer *in vivo* and, consequently, a robust controlled drug release.

Table 3: Bilayer F_{HE6005} kinetic analysis results.

Models	Kinetic constant	Coefficient of determination (R^2)
First-order	0.0914	0.9989
Zero-order	0.0041	0.9729
Higuchi	22.4790	0.9963
Hixson-Crowell	0.1321	0.9593
Korsmeyer-Peppas		
Kinetic constant	Coefficient of determination (R^2)	Exponent (*n*)
1.0133	0.9992	0.66

Table 4: Bilayer A1 kinetic analysis results.

Models	Kinetic constant	Coefficient of determination (R^2)
First-order	0.0877	0.9997
Zero-order	0.0040	0.9825
Higuchi	22.3140	0.9971
Hixson-Crowell	0.1389	0.9569
Korsmeyer-Peppas		
Kinetic constant	Coefficient of determination (R^2)	Exponent (*n*)
0.0560	0.9999	0.72

3.8 In vivo pharmacokinetic study

The gastroretentive bilayer F_{HE6005} and bilayer A1 tablets were administered to four Beagle dogs after a meal. This choice was made as gastric pH is higher during the stomach fed state, which could potentially

promote the gastric retention of the formulations, based on the SEM and viscosity findings from the *in vitro* buoyancy testing in media of different pH. Furthermore, the presence of food in the stomach disrupts the MMC and housekeeper waves, thereby leading to a prolonged gastric residence time of the stomach contents [18, 76-78].

Figure 7 illustrates the pharmacokinetic profiles of MT-1207 in the dog plasma after oral administration of one MT-1207 30 mg bilayer F_{HE6005} tablet, one MT-1207 30 mg bilayer A1 tablet, and three MT-1207 10 mg immediate-release tablets. The pharmacokinetic parameters recorded on the respective dosing regimens in Beagle dogs can be seen in Table 8. As per preliminary data, the minimum effective concentration of MT-1207 in plasma has been determined to be approximately 1 ng/mL (Internal communications with Shenyang Haiwang Biotechnology Co Ltd; unreferenced). Sustained-release pharmacokinetic profiles of MT-1207 in dog plasma were recorded after oral administration of bilayer F_{HE6005} and bilayer A1 tablets. There was a statistically significant difference in the $t_{1/2}$ (one-way ANOVA, $p < 0.05$) and T_{max} values (one-way ANOVA, $p < 0.01$) recorded between the different dosing regimens with pairwise comparisons demonstrating significant differences between each of the gastroretentive bilayer dosing regimens and the immediate-release one ($t_{1/2}$: *post hoc* Bonferroni, $p < 0.05$, T_{max}: *post hoc* Bonferroni, $p < 0.01$). There was no statistically significant difference between the $t_{1/2}$ values reported for the gastroretentive dosing regimens (*post hoc* Bonferroni, $p > 0.05$). Furthermore, there was no statistically significant difference in the C_{max} values recorded between the different dosing regimens (one-way ANOVA, $p > 0.05$). This could be attributed to the fact that, during fed state conditions, gastric emptying rates tend to be lower, compared to the fasted state [18, 78-80]. Consequently, the amount of MT-1207 released in the stomach during the fed state was probably emptied gradually and at a slower rate, thereby resulting in smaller peak plasma concentration values. Additionally, the abovementioned parameter values were not statistically different to those recorded after oral administration of A1 tablets in our previous study (one-way ANOVA, $p > 0.05$) [17]. With regard to clearance, there was no statistically significant difference between the values recorded for the different dosing regimens in the present pharmacokinetic study (one-way ANOVA $p > 0.05$).

Table 5: Pharmacokinetic parameters of MT-1207 after oral administration of a single MT-1207 30 mg gastroretentive bilayer F_{HE6005} tablet (F_{HE6005} o.d.), a single MT-1207 30 mg gastroretentive bilayer A1 tablet (Bilayer A1 o.d.), and three MT-1207 10 mg immediate-release tablets administered at the same time (3 × 10 mg IR o.d.) to Beagle dogs (n=4).

Dosing Regimen	$t_{1/2}$ (h)	T_{max} (h)	C_{max} (ng/mL)	AUC_{0-24h} (h*ng/mL)	$AUC_{0-\infty}$ (h*ng/mL)	V_d (L/kg)	CL (L/h/kg)	MRT (h)
F_{HE6005} o.d.	5.10±1.37	6.00±1.63	22.29±11.50	226.6±97.9	232.7±100.4	78.1±23.2	11.4±5.4	9.23±1.05
Bilayer A1 o.d.	4.76±1.13	6.50±1.00	31.74±18.92	288.9±167.5	295.1±169.7	60.6±19.1	9.7±4.9	8.71±0.32
3 × 10 mg IR o.d.	2.47±0.09	2.25±1.26	49.72±33.70	283.6±183.2	295.1±189.9	78.9±21.2	11.6±8.9	5.36±1.01

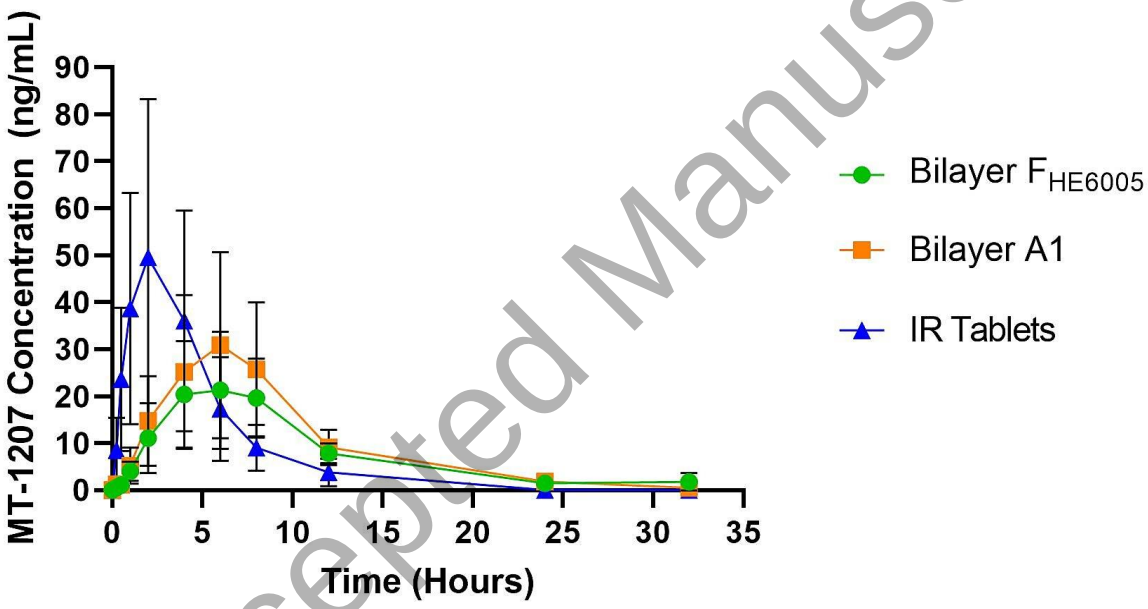


Figure 6: Plasma concentration of MT-1207 in Beagle dog after oral administration of bilayer F_{HE6005} tablet o.d., bilayer A1 tablet o.d., and 3 × 10 mg IR tablet o.d. (n = 4).

The bioequivalence between the gastroretentive and immediate-release tablets was investigated by calculating the AUC_{0-24h} ratio of each. The ratio values calculated for the bilayer F_{HE6005} and bilayer A1 formulations were 92.31 ± 29.33 % and 112.35 ± 35.20 %, respectively. These values fell within the 80.00 – 125.00 % range outlined in the EMA guidelines for the AUC_{0-t} ratios that allows for bioequivalence to be established between the test and reference products [81]. This is an indication that there can be bioequivalence

between the gastroretentive formulations and the immediate-release tablets, and that MT-1207 was fully released from the former. However, the relatively high standard deviations linked to the $AUC_{0\text{-}24h}$ ratio values need to be considered. This variation in the AUC values between the different Beagle dogs could be attributed to the built-in subject variability of this animal model. Previous studies have demonstrated considerable inter- and intra-animal variations in the gastric pH of different Beagle dogs [82, 83]. Such variations in the present study could potentially have noticeable effects on the solubility of MT-1207 in the stomach contents, thereby introducing inter-subject variability in its solubilisation, release, and consequent absorption and bioavailability.

The distribution of MT-1207 in the Beagle dog body was reflected by the V_d and MRT parameters. V_d indicated the extent of distribution of MT-1207 in the peripheral tissues, while MRT indicated the average time that the drug resided in the body. There was no statistically significant difference in the V_d values recorded for the different dosing regimens (one-way ANOVA, $p > 0.05$). All V_d values were sufficiently high to indicate extensive distribution of the API in the peripheral tissues. Additionally, there was a statistically significant difference between the MRT values of the three different dosing regimens (one-way ANOVA, $p < 0.001$) with pairwise comparisons demonstrating statistically significant differences between each of the gastroretentive dosing regimens and the immediate-release one (*post hoc* Bonferroni, $p < 0.001$). This indicates that the time during which the drug resided in the body and, therefore, potentially provided antihypertensive action was significantly prolonged when MT-1207 was administered in the form of gastroretentive tablets, instead of immediate-release tablets. Furthermore, the MRT values of MT-1207 after administration of the gastroretentive formulations were significantly longer than the MRT value recorded after administration of single-layer A1 tablets in our previous study (one-way ANOVA, $p < 0.001$; *post hoc* Bonferroni, $p < 0.05$ for pairwise comparisons). This indicated the potential of prolonged pharmacological activity of MT-1207 when formulated into gastroretentive bilayer tablets, compared to single-layer A1 tablets [17].

The pharmacokinetic study demonstrated the ability of the gastroretentive formulations to provide a sustained release of MT-1207 *in vivo*, as indicated by the significantly prolonged $t_{1/2}$, T_{max} and MRT values, compared to the respective ones recorded after administration of the immediate-release tablets [84]. Furthermore, the gastroretentive formulations were able to fully release the API within 24 hours. These findings, alongside the significant prolongation of MRT, compared to single-layer A1 tablets, indicate the significant role of the gastroretentive layer in retarding the release of MT-1207, potentially due to the reduced surface area of the drug layer that was exposed to the stomach contents, and prolonging the residence of the drug *in vivo*. Furthermore, the gastroretentive layer potentially ensured robust gastric retention that was long enough to provide a 24-hour

complete release and absorption of MT-1207 in the upper part of the GIT. *In vivo* monitoring of the formulations could give a deeper insight into the ability of the gastroretentive formulations to remain in the Beagle dog stomach over 24 hours [41, 78], although, the pharmacokinetic study results provided promising indication towards this occurring. Additionally, the presence of ethylcellulose had only a minor effect on drug release *in vivo* which was in alignment with the *in vitro* release testing findings.

Another important factor that needs to be considered is the significantly higher average MT-1207 $t_{1/2}$ value of 7.28 – 7.38 hours recorded in humans after administration of 20 – 40 mg of the drug [15]. Since MT-1207 is mainly biotransformed via S-methylation in the liver, this inter-species discrepancy in the $t_{1/2}$ of the drug could be due to the higher hepatic metabolism of Beagle dogs, compared to humans [85]. Therefore, it can be concluded that both gastroretentive bilayer F_{HE6005} and bilayer A1 formulations are promising for the once-a-day oral delivery of MT-1207.

4 Conclusions

The aim of the present study was to develop at least one effective gastroretentive tablet formulation for the once-a-day oral delivery of MT-1207. The novel gastroretentive layer that was used in the formulations was easy to prepare and provided an immediate and robust 24-hour tablet buoyancy *in vitro* and *in vivo* irrespective of the drug layer. The presence of the gastroretentive layer was also the main factor contributing to the sustained release of MT-1207 from the tablets. Additionally, *in vitro* buoyancy testing of the optimised ethylcellulose-containing bilayer tablets in media of different pH demonstrated the importance of assessing the effect of pH on the buoyancy of non-effervescent gastroretentive systems which could help design better dosing regimens, regarding administration of the dosage form with or without a meal. Even though the gastric retention capability of this gastroretentive layer should further be assessed in an *in vivo* monitoring study, the combination of hydrophilic and hydrophobic polymers could have promising gastroretentive tablet applications for the once-a-day delivery of different drugs in the form of gastroretentive bilayer and/or single-layer tablets.

The gastroretentive bilayer F_{HE6005} and bilayer A1 formulations are deemed promising for the once-a-day oral delivery of the drug candidate MT-1207 and will, therefore, be forwarded to clinical development.

Ethics approval and consent to participate: The *in vivo* study complied with the principles of Laboratory Animal Care and was approved by China Pharmaceutical University Animal Management and Ethics Committee. The rest of the experiments conducted were *in vitro*.

Consent for publication: All authors read and approved the final manuscript for publication.

Availability of data and materials: N/A

Declaration of interest: The authors declare that Shenyang Haiwang Biotechnology Co., Limited which funded the present study is the patent holder of MT-1207 agent.

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Authors' contributions: **Napoleon-Nikolaos Vrettos**: Conceptualisation, Data curation, Formal analysis, Investigation, Methodology, Project administration, Validation, Visualisation, Writing - original draft; **Peng Wang**: Conceptualisation, Resources, Methodology, Writing - Review & Editing, Funding acquisition; **Yuhan Wang**: Data curation, Writing - Review & Editing; **Clive J. Roberts**: Conceptualisation, Methodology, Project administration, Supervision, Visualisation, Writing - Review & Editing; **Jinyi Xu**: Writing - Review & Editing, Funding acquisition; **Hong Yao**: Methodology, Writing - Review & Editing; **Zheying Zhu**: Conceptualisation, Methodology, Project administration, Supervision, Resources, Visualisation, Writing - Review & Editing, Funding acquisition.

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