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RESEARCH PAPER

Temozolomide-Based Dry Powder Formulations for Lung Tumor-Related Inhalation Treatment

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ABSTRACT

Purpose Temozolomide dry powder formulations for inhalation, performed with no excipient or with a lipid or lactose coating, have been evaluated.

Methods The particle size of raw temozolomide in suspension was reduced by a high-pressure homogenizing technique, and the solvent was evaporated by spray-drying to obtain a dry powder. The physicochemical properties of this powder were evaluated and included its crystalline state, thermal properties, morphology, particle size and moisture and drug content, and these properties were determined by X-ray powder diffraction, differential scanning calorimetry, scanning electron microscopy, laser light scattering, thermogravimetric analysis and high-performance liquid chromatography, respectively. The aerodynamic properties and release profiles were also evaluated using a multistage liquid impinger and a modified USP type 2 dissolution apparatus adapted for inhaler products, respectively.

Results The dry powder inhalation formulations had a high temozolomide content that ranged from 70% to 100% in the crystalline state and low moisture content. Aerodynamic evaluations showed high fine-particle fractions of up to 51% related to the metered dose. The dissolution profile revealed a similarly fast temozolomide release from the formulations.

Conclusions Dry temozolomide powder formulations, based on the use of acceptable excipients for inhalation and showing good dispersion properties, represent an attractive alternative for use in local lung cancer therapy.

KEY WORDS aerosol chemotherapy · DPI · lung cancer · pulmonary delivery · temozolomide

ABBREVIATIONS

DLPC	1,2-dilauroyl-sn-glycero-3-phosphocholine
DMPC	1,2-dimyristoyl-sn-glycero-3-phosphocholine
DPI	dry powder inhaler
DPPC	dipalmitoyl phosphatidylcholine
DSC	differential scanning calorimetry
FPD	fine particle dose
FPF	fine particle fraction
HPH	high-pressure homogenizing
HPLC	high-performance liquid chromatography
HPMC	hypromellose
IV	intravenous
MMAD	mass median aerodynamic diameter
MsLI	multi-stage liquid impinger
MTIC	5-(3-methyltriazen-1-yl)imidazole-4-carboxamide
NGI	next generation impactor
NSCLC	non-small cell lung cancer
P90H	phospholipon 90H
SCLC	small cell lung cancer
SEM	scanning electron microscopy
SLF	simulated lung fluid
TGA	thermogravimetric analysis
TMZ	temozolomide
XRPD	X-ray powder diffraction

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INTRODUCTION

Lung cancer has remained the leading fatal cancer in men and women for the last several decades in Western countries (1). Non-small cell lung cancers (NSCLCs) and small cell lung cancers (SCLCs) represent ~85% and ~15% of primary lung cancers, respectively (2). In addition, the lungs are also a common site for metastatic processes from prostate, breast, colorectal, kidney, head and neck carcinomas as well as from sarcomas and melanomas (3,4). The treatment of NSCLCs depends on the stage of the disease and usually involves a combination of surgery, radiotherapy and/or chemotherapy (2). Chemotherapy could be used in the early stages as a neoadjuvant treatment to reduce the tumor size before surgery, as an adjuvant therapy to radiotherapy or surgery and as a palliative therapy for advanced and metastatic diseases (2). Currently, non-specific and non-selective cytotoxic chemotherapies are delivered by infusion via the intravenous (IV) route for several hours and cause severe systemic toxicities to the patient. These toxicity-related features require interruption of the treatment to allow normal tissue to recover, and this process occurs in parallel with tumor cell repopulation in various organs (5). In addition and because of this dose-limiting toxicity, only a modest increase in patient survival time occurs, because effective therapeutic concentrations of the cytotoxic drugs may not be reaching the tumor site via the infusion route (5,6). Chronic IV treatments to cancer patients, including NSCLC patients, are also associated with multiple adverse events, including damaged veins, infection at the catheter introduction site or air embolisms via the intravenous line (7).

Delivery of chemotherapeutic agents, including cytotoxic drugs, via the pulmonary route for the treatment of lung tumors has been investigated since 1968 (8). The development of inhaled chemotherapy has been limited because of conventional chemotherapy-induced lung toxicity in 10% to 20% of the cases. In addition, some antineoplastic agents are associated with pulmonary toxicity (9). Despite this limiting factor, an increasing number of preclinical studies and early clinical trials demonstrates the clinical potential and feasibility of this approach by achieving a high therapeutic ratio and sharp decrease in severe systemic side effects (5,10). The most adverse events observed when treating lung cancer patients through the inhalation route are related to the direct effects of the inhaled drug on the upper and lower respiratory tracts, and they primarily depend on the dose and the drug administered (10). Consequently, lung toxicity must be evaluated for each drug considered for inhalation treatment.

In most reports in the literature, the inhalation device used to deliver cytotoxic chemotherapeutics is an air jet nebulizer (5,11–13). However, these devices display many disadvantages, including being cumbersome, requiring additional tubing and mouthpieces and requiring compressed air and/or oxygen sources. Moreover, they present long administration time, high cost, risk of device contamination, and facial and environmental exposure. These devices display, in general, low efficiency and poor delivery reproducibility and require regular maintenance (14). Another approach for delivering cytotoxic chemotherapeutics to the lung could be through a formulation of a dry powder for inhalation and the selection of an appropriate device that is activated and driven by the patient's inspiratory flow during a short administration time. Dry powder inhalers (DPIs) present many advantages compared to liquid nebulizer systems. One advantage is that DPI-based formulations are in a solid state, which is more stable for long-term storage and better adapted to poorly water-soluble drugs, such as conventional cytotoxic chemotherapeutics. Moreover, the administration time by DPI takes several seconds, or several minutes if multiple doses are required, in comparison with several hours if the drug is administered via the conventional parenteral treatments. Furthermore, the devices can be easily transported by patients, are less expensive, require less maintenance and can be manufactured as disposable inhalers to limit device and environmental contamination. The successful delivery of therapeutic aerosols directly into the desired airways regions is dependent on a combination of the aerodynamic and physicochemical characteristics of the inhaled particles, the performance of the inhaler device, the patient's inhalation dynamics and lung physiology/disease (5,15).

Particle size is the most important design variable in an aerosol or dry powder formulation. Shape, density, electrical charge and hygroscopicity are also important formulation variables (15). Moreover, in the case of DPIs, micronized particles are generally very cohesive and exhibit poor flow properties, which require improvements to be made by means of particle engineering (e.g., optimization of particle size) and/or excipients (e.g., lactose or lipids) (15). In this study, we produced and evaluated temozolomide (TMZ)-based dry powder formulations by reducing TMZ particle size and adding no or a low proportion of excipients to deliver a high drug dose to the pulmonary tract. Because only a low number of excipients are authorized by the FDA for inhalation use (15), the excipients chosen were lactose, phospholipids and cholesterol, which are well tolerated by the respiratory tract (15). The excipients are used to improve the aerodynamic characteristics of the particles by decreasing cohesion and increasing their flowability. Moreover, these excipients could influence the dissolution profiles of the powders in the lungs due to their hydrophilic, hydrophobic or amphiphilic nature. TMZ is slightly soluble in water, which could present dissolution problems in the lungs. In

addition, the aerodynamic characteristics were optimized to deliver chemotherapeutic drugs to tumors that could be located in the conducting and the respiratory zones of the lungs (16,17).

We chose TMZ as the model drug for this study because it is clinically active against cancers associated with extremely poor prognoses, such as glioblastomas and melanomas, and in experimental cancer models, including pre-clinical models of NSCLC, breast, prostate, ovarian, and head and neck cancers (18). In addition, TMZ, as a cytotoxic cancer treatment agent, presents an acceptable safety profile with adverse reactions mainly characterized by myelosuppression, which rarely requires discontinuation of therapy (19). In addition, only rare respiratory adverse reactions have been reported (20). TMZ is an alkylating agent that induces sustained pro-autophagic effects in cancer cells, which is a feature that then leads to apoptosis in these cancer cells (18). TMZ has also recently been evaluated in a few clinical studies (Phase I and II) for NSCLC patients (21–23, http://www.clinicaltrials.gov website). TMZ induces sustained pro-autophagic effects in cancer cells and the ultimate consequence (but not a direct cause) of apoptotic cell death. Therefore, TMZ could overcome the intrinsic resistance of a number of cancer types (NSCLCs, glioblastomas, melanomas, pancreas cancers and esophageal cancers) to cytotoxic drugs that induce pro-apoptotic effects as a direct effect of their mechanism of anticancer action (18,24). We recently demonstrated the *in vivo* therapeutic benefits of inhaled TMZ in a mouse melanoma pulmonary pseudometastatic model (25) that displays significant resistance to pro-apoptotic stimuli (26). The current study aims to develop dry powder formulations for use in humans with physico-chemical properties promoting long-term stability, usable aerodynamic behaviors and relatively rapid dissolution profiles.

MATERIALS AND METHODS

Materials

TMZ was supplied from Shilpa Medicare Limited (Raichur, India), and 1,2-dilauroyl-sn-glycero-3-phosphocholine (DLPC) and 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) were purchased from the NOF Corporation (Hyogo, Japan). Cholesterol was purchased from Bufa (Uitgeest, the Netherlands). Phospholipon 90H (P90H) and hydrogenated soy lecithin, with more than 90% hydrogenated phosphatidylcholine and 15% dipalmitoyl phosphatidylcholine, were donated by Nattermann Phospholipids (Koln, Germany). Dipalmitoyl phosphatidylcholine (DPPC) was purchased from Lipoid (Ludwigshafen, Germany), and α-lactose monohydrate (Lactose 450 Mesh) was supplied from DMV (Veghel, the Netherlands). Potassium phosphate was purchased from Merck (Darmstadt, Germany), as were HPLC-grade acetonitrile, glacial acetic acid, hydrochloric acid and isopropanol. All chemicals used were of analytical grade.

Methods

Hazardous Drug Procedures

TMZ is a hazardous drug, and procedures were used to protect the manipulator and the environment. Personal protective equipment included longer, powder-free latex gloves that were worn under the gown cuff and a second pair of powder-free latex gloves that were worn over the gown cuff. The latter were removed every hour, at most, or immediately if they were punctured or stained with the product. A protective Tyvek® disposable gown (DuPont, Mechelen, Belgium) was worn and was not permitted to be worn outside the preparation area. A respirator was used with a FFP3 particle-filtering face piece (3M, Cergy-Pontoise, France). Eyeglasses, with temporary side shields, were used to protect the eyes. All gowns, gloves and disposable materials were disposed of as hazardous drug waste.

The preparation work area was composed of two flow cabinets (Protec I and Protec II, ADS Laminaire, Paris, France) that were designed for our application with air circulating through high-efficiency particulate air (HEPA) filters before being eliminated outside of the building.

Preparation of the TMZ Dry Powder Formulations—High-Pressure Homogenizing (HPH) and Spray-Drying

The theoretical composition of the suspensions used to prepare the dry powder formulations F1, F2, F3 and F4 by spray-drying is described in Table I.

A TMZ particle size-reduction step was necessary to obtain stable suspensions (in term of homogeneity) and an adequate inhalation particle size range of 1–5 μm during the spray-drying step. First, TMZ (5% m/v) was dispersed in isopropanol or in a phosphate buffer (pH 5.0) containing dispersions of DLPC and DMPC by a high-speed stirrer-homogenizer composed of an X620 motor coupled to a T10 dispersing tool (CAT M. Zipperer, Staufen, Germany). The procedure involved homogenizing for 10 min at a speed of 24,000 rpm in an ice bath to prevent sample temperature increase. Second, an EmulsiFlex-C5 high-pressure homogenizer (Avestin Inc., Ottawa, Canada) was used. It applies pre-milling, low-pressure homogenizing cycles (15 cycles at 4,000 PSI and 10 cycles at 12,000 PSI) to avoid blocking the homogenizing gap before performing

Table I Theoretical Composition of Formulations (F1, F2, F3 and F4) Before and After Spray-Drying.

	Theoretical composition of the suspensions before spray-drying	Theoretical composition of the dry powders after spray-drying
F1	TMZ 5% Isopropanol ad100%	TMZ 100%
F2	TMZ 5%	TMZ 95%
	P90H 0.197%	P90H 1.25%
	Cholesterol 0.066% Isopropanol ad 100%	Cholesterol 3.75%
F3	TMZ 5%	TMZ 48.544%
	DLPC 1.4%	DLPC 13.592%
	DMPC 1.4%	DMPC 13.592%
	Lactose 2.5% Phosphate buffer pH 5.0 ad 100%	Lactose 24.272%
F4	TMZ 5%	TMZ 43.29%
	DLPC 1.4%	DLPC 12.12%
	DMPC 1.4%	DMPC 12.12%
	Lactose 3.75% Phosphate buffer pH 5.0 ad 100%	Lactose 32.47%

high-pressure homogenizing cycles (20 cycles at 20,000 PSI). The process was carried out using a "closed loop" approach, and stirring was maintained at 8,000 rpm in the sample reservoir to avoid sedimentation of the particles in suspension. All experiments were performed using a heat exchanger downstream of the homogenizing valve to maintain a relatively low and constant temperature of $2.5 \pm 1°C$ for the suspension with isopropanol and $10 \pm 1°C$ for the suspension in phosphate buffer. By maintaining these temperatures, evaporation and heating of the suspensions during the process were limited. Third, TMZ content in the suspensions was determined by high-pressure liquid chromatography (HPLC), and an adequate amount of P90H (1.25% of TMZ weight) and cholesterol (3.75% of the TMZ weight) for F2 or lactose for F3 (50% of the TMZ weight) and F4 (75% of the TMZ weight) was added to the suspensions and dissolved by stirring. Finally, the suspensions were spray-dried using a B-290 Mini Spray Dryer (Büchi Laboratory-Techniques, Flawil, Switzerland) at a fixed relative humidity (50–60%) with a B-296 dehumidifier (Büchi Laboratory-Techniques, Flawil, Switzerland). While the suspensions were stirring, they were pneumatically pumped into the drying chamber at a rate of 3.4 g/min for F1 and F2, and 2.2 g/min for F3 and F4. The suspensions were atomized through a 0.7 mm-diameter nozzle with a 1.5-mm nozzle cap using compressed air at 500 l/h. The drying airflow occurred at a rate of 35 m^3/h, and it was heated to 70°C for F1 and F2 and to 130°C for F3 and F4. In these conditions, the outlet temperatures were 35°C for F1 and F2 and 60°C for F3 and F4. The spray-dried powders were blown through a cyclone separator and collected in a container. The process yield was about 40% for F1, 60% for F2 and, 50% for F3 and F4. The volumes of isopropanolic suspension and aqueous spray-dried suspension were 50 ml and 90 ml, respectively, following the conditions described above. The dried formulations were stored in a desiccator at ambient temperature. The amount of the coating was calculated as the percentage expressed to the total solids from the results obtained from the determination of TMZ in the different formulations.

Particle Size—Laser Light Scattering

The particle size distribution properties of raw TMZ, the dry powder formulations and the corresponding suspensions after the size-reduction step were measured by a Malvern Mastersizer 2000 laser diffractometer with a Hydro 2000 wet sampling system (Malvern Instruments Ltd., Worcestershire, UK). The dried formulations were analyzed after redispersion in isopropanol for F1 (TMZ is less soluble in isopropanol (~0.1 mg/ml at 25°C) than in water (~3 mg/ml at 25°C)), F3 and F4 (to avoid the dissolution of the lactose coating), and in phosphate buffer (pH 5.0) for F2 (to avoid the dissolution of the lipid coating). To analyze the dispersed samples, we used a refractive index of 1.475 and an absorption index of 1.50. The dispersant media was isopropanol (saturated with TMZ) with a refractive index of 1.390 or phosphate buffer (pH 5.0) (saturated with TMZ) with a refractive index of 1.330. Malvern Mastersizer software Version 5.54 (Malvern Instruments Ltd. Worcestershire, UK) was used to characterize the volume median particle size (d(v;0.5) in μm) and two additional parameters, d(v;0.1) and d(v;0.9) (the size, in microns, at which 10% and 90% of the particles are

smaller than the remaining distribution, respectively). Three runs of five measurements were performed for each sample.

Size and Morphology—Scanning Electron Microscopy (SEM)

The size and the morphology of raw TMZ, the dry powder formulations (F1, F2, F3 and F4) and the lactose spray-dried formulations (at the same conditions as F3 and F4) were determined using a Philips ESEM XL30 FEG scanning electron microscope (FEI, Eindhoven, the Netherlands) following gold coating (35 mA for 90 s at 5.10^{-2} mbar under argon).

Crystalline State—X-Ray Powder Diffraction (XRPD)

XRPD is a powerful and widely used tool for crystalline-state characterization. Diffraction patterns of raw TMZ and the dried formulations (F1, F2, F3 and F4) were determined using a Siemens D5000 diffractometer (Siemens, Munich, Germany) with a Cu line as the source of radiation (WL1 = 1.5406 A, WL2 = 1.54439 A) and standard runs using a 40-kV voltage, a 40-mA current and a scanning rate of 0.02°/min over a 2 θ range of 2–70°.

Thermal Properties—Differential Scanning Calorimetry (DSC)

The thermal properties of raw TMZ and the dried formulations were investigated by means of a Q2000 differential scanning calorimeter (TA Instruments, Zellik, Belgium) with a refrigerated cooling system (TA Instruments, Zellik, Belgium) and Universal Analysis 2000 version 4.4A software (TA Instruments, Zellik, Belgium). The amount of product analyzed ranged from 1 to 3 mg and was placed in Tzero aluminum pans. The heat runs for each sample were set from 0°C to 230°C at 5°C/min using nitrogen as a blanket gas.

Moisture Content Determination—Thermogravimetric Analysis (TGA)

The amount of residual water in raw TMZ and the dried formulations was assessed by TGA with a Q500 apparatus (TA Instruments, Zellik, Belgium) and Universal Analysis 2000 version 4.4A software (TA Instruments, Zellik, Belgium). Runs in triplicate were set from 25°C to 300°C at a heating rate of 10°C/min at high resolution, which controlled the heating rate in response to the measured rate of weight change in order to separate the "free" surface water (moisture) and the bound water. Samples weighed approximately 10 mg. The moisture level was determined by the weight loss obtained between 25°C and 125°C.

TMZ Determination—High-Performance Liquid Chromatography (HPLC)

TMZ determination of the dry powder formulations, the aerodynamic particle size analysis and the release profiles was performed using a validated HPLC method. The chromatographic system (HP 1200 series, Agilent Technologies, Brussels, Belgium) was equipped with a quaternary pump, an auto sampler and a diode array detector. The separations were performed on a reverse-phase Hypersil Gold C-18 column (5 µm, 250 mm × 4.6 mm) (Thermo Fisher Scientific, Waltham, USA). The mobile phase consisted of 0.5% v/v aqueous acetic acid-acetonitrile (90:10 v/v), which was delivered at a flow rate of 1.0 ml/min. The quantification was performed at 329 nm. The calibration curve was linear in the 1–250 µg/ml range. The TMZ samples and calibration standards were diluted in the mobile phase or in 0.5% acetic acid in deionized water (v/v). The volume injected was 10 µl, the temperature was set at 25°C and the analysis time was 10 min.

Aerodynamic Particle Size Analysis—Multi-Stage Liquid Impinger (MsLI)

The fine particle dose (FPD) and aerodynamic particle size distribution characterized by mass median aerodynamic diameter (MMAD) were determined by following the procedure for powder inhalers using Apparatus C (a MsLI (Copley Scientific, Nottingham, United Kingdom)), as described for the aerodynamic assessment of fine particles in the European Pharmacopeia 6.0. The dry powder inhalation device was an Axahaler® (SMB, Brussels, Belgium). Three N°3 hypromellose (HPMC) capsules (Capsugel®, Colmar, France) were filled with about 20 mg of the dried formulations for each assay. Three assays for each formulation were performed at ambient temperature and humidity. The airflow rate was determined by the uniformity test of the delivered dose for obtaining a pressure drop across the inhaler of 4 kPa (100 l/min). The cut-off diameters at this test flow rate for the MsLI were 5.27, 2.40 and 1.32 µm between stages 2 to 3, 3 to 4 and 4 to 5, respectively. The test airflow duration was the time taken to draw a volume of 4 l of air from the mouthpiece of the inhaler and through the MsLI at the test flow rate (2.4 s). The flow rate was measured by a DFM3 flow meter (Copley Scientific, Nottingham, United Kingdom). The solvent used to dissolve the active substance in the four upper stages was 0.5% acetic acid in deionized water (v/v). Drug deposition in the device (mouthpiece adapter, inhaler and capsule), the induction port simulating the throat, the four stages and the filter (stage 5) of the MsLI were determined by HPLC analysis. The total mass of the active substance collected for each MsLI was in the

range of 75–125% of the average TMZ content. FPD is the mass of the active substance with aerodynamic diameters smaller than 5 μm. The FPD was determined by interpolation from the cumulative mass *versus* the cut-off diameter of the respective stage. The fine particle fraction (FPF) was expressed as a percentage of the metered dose but not of the delivered dose. The metered dose is the total dose recovered from the device (capsule and inhaler), the throat and the stages of the impinger, and the emitted dose is the total powder mass exiting the capsule and device.

Inhaled Dry Powder Release Profile—Optimized Dissolution Test for Inhaler Products

A USP 33 type II (paddle method) dissolution apparatus (Erweka DT6, Heusenstamm, Germany), adapted to dry powders for inhalation, was used to conduct the release studies for TMZ from the formulations (F1, F2, F3, and F4). Placed in the bottom of this dissolution vessel was a membrane cassette (polycarbonate membrane (0.1 μm-diameter pore) (Copley Scientific Limited, Nottingham, UK)) with ~5 mg of TMZ, a stainless steel membrane holder (Copley Scientific Limited, Nottingham, UK) with a quick release dose plate, a dose collection body and a sealing ring.

The dose was collected into the membrane cassette with a Next Generation Impactor (NGI) (Copley Scientific Limited, Nottingham, UK), and the dose collection body was fixed at the quick release dose plate at stage 3. An Axahaler® (SMB, Brussels, Belgium) DPI device was used containing a N°3 HPMC capsule (Capsugel®, Colmar, France) filled with an appropriate dose of each formulation (~30 mg for F1 and F2, ~70 mg for F3 and ~50 mg for F4). After dispersal into the NGI through the appropriate induction port at a flow rate of 60 l/min for 4 s, this device was used to obtain about 5 mg of TMZ at stage 3. The dose collection body was then removed from the quick release collection plate, and a membrane was placed on top of it and sealed in place with the sealing ring. The membrane cassette was then placed into the dissolution vessel.

The dissolution conditions included the following: (i) a paddle operating speed of 75 rpm, (ii) a distance of 25 mm between the bottom of the blade and the inside base of the vessel, (iii) a dissolution medium volume of 300 ml, which was composed of a simulated lung fluid (SLF) as described by Sdraulig *et al.* (27) and fixed at pH 5.0 with 32% HCl to guarantee the stability of TMZ, and (iv) a dissolution medium temperature maintained at 37.0 ± 0.2°C. The dissolution tests were carried out in triplicate for each formulation, and the percentages of dissolved TMZ were determined by HPLC analysis at pre-selected time intervals up to 180 min. The concentration determined at 180 min was considered to be that for 100% TMZ dissolution.

Statistical Analyses

The similarity of dissolution profiles was determined using the similarity factor (f_2), as recommended by the Food and Drug Administration's Guidelines for Industry. f_2 is calculated by the method described by Shal *et al.* (28) and must be higher than 50 to assess the similarity between two dissolution profiles.

RESULTS AND DISCUSSION

The approved and conventional TMZ treatment regimen for recurrent gliomas is a daily dose of 150–200 mg/m^2 of body surface area by infusion over 90 min or by mouth for 5 days and repeated every 28 days (29). Our previous *in vivo* experimental study used a mouse melanoma pseudometastatic lung model, and we obtained the same efficacy in terms of median survival period when comparing TMZ administered through inhalation to TMZ administered intravenously (25). In addition, the local inhaled therapy resulted in long-term mouse survival with an almost complete eradication of lung tumors (25). Considering an extrapolation of this treatment to humans with no reduction in the dose achieved, a dose of 320 mg for an adult of 60 kg must be delivered by inhalation. The delivery of a high dose by inhalation is a challenge; therefore, the development of formulations that minimize the amount of excipient and optimize the aerodynamic and dissolution properties of the inhalation powder is important. Different dry powder formulations for inhalation, either with no excipient or with a lipid or lactose coating, were developed with the aim of optimizing these parameters and reducing the administered inhalation dose.

Production of the Dried Formulations

The HPH process reduced the TMZ particle size from a d (v;0.5) value of 21.3 μm to 1.5 μm, and 99.6% of the particles displayed a size spread of 1–5 μm, as compared to only 5.0% before the size reduction process (Table II). After spray-drying the suspension and evaporating the solvent (isopropanol for F1 and F2 and water for F3 and F4), the TMZ content was determined for each dry powder formulation (Table III). The actual F1 and F2 TMZ content corresponds to what was expected according to the amount of drug introduced into the initial suspensions (i.e., the theoretical TMZ content). However, the F3 and F4 formulations showed higher TMZ content than the theoretical composition. Therefore, it seems that the lactose coating

Table II The Particle Size Characteristics of Raw TMZ, TMZ Suspensions After HPH Processing and the Dry Powder Formulations (F1, F2, F3 and F4). The Percentage <5 μm (%) and the d(0.5) (μm) (Mean ± S.D., $n=3$) Were Measured with a Mastersizer 2000® Laser Diffractometer. The FPF (%), MMAD (μm) and FPD (mg) Were Determined Using an MsLI at 100 l/min for 2.4 s with an Axahaler® (mean ± S.D., $n=3$).

	Laser light scattering		MsLI		
	% <5 μm (%)	d(v;0.5) (μm)	FPF (%)	FPD (mg)	MMAD (μm)
Raw TMZ	5.0 ± 0.1	21.3 ± 0.4	/	/	/
Isopropanolic TMZ suspension after HPH	99.64 ± 0.04	1.50 ± 0.01	/	/	/
Aqueous TMZ suspension after HPH	99.3 ± 0.2	1.53 ± 0.06	/	/	/
F1	99.17 ± 0.07	1.65 ± 0.01	49 ± 4	12 ± 1	2.9 ± 0.3
F2	97.7 ± 0.4	1.77 ± 0.07	51 ± 2	12 ± 1	3.1 ± 0.2
F3	76 ± 3	1.97 ± 0.09	26 ± 2	5.6 ± 0.4	4.57 ± 0.04
F4	72 ± 2	2.75 ± 0.09	41 ± 4	9.0 ± 0.8	3.8 ± 0.2

crumbled away during the drying process. Consequently, the actual TMZ content is the value that should be used in *in vitro* and future *in vivo* evaluations.

Physicochemical Characterization of Dried Formulations

The XRPD patterns (Fig. 1) show that the particle size reduction and the spray-drying process did not affect the crystalline form of TMZ. The maintenance of the initial crystalline state of a drug after HPH and spray-drying processing has already been demonstrated for other drugs, such as nifedipine (30) and tobramycin (31). Each diffraction peak observed for the dry powder formulations corresponds to those obtained for the raw TMZ. The F3 and F4 formulations showed additional peaks at 12.5, 16.4, 20.0 and 20.9°, corresponding to α-lactose monohydrate (32). No diffraction peaks characterizing cholesterol or P90H were observed for F2. This could be explained by the lack of method sensitivity and the limited coating for the lipid-coated formulation (4% of the TMZ weight) (33). The moisture content, as evaluated by TGA, was very low (below 1%, Table III), and the lowest content was seen in F1 and F2. This method was used to determine the weight loss observed between 25 and 125°C, which corresponds to the residual water or solvent in the powder. The only solvent residues found were water (for all dry powders) and isopropanol (for F1 and F2). The latter is generally found at very low levels (<250 ppm) when a spray-drying technique is used for the preparation of solid lipid formulations (34), and this is generally determined by gas chromatography. The presence of the initial crystalline form and low moisture content are both important to promote the long-term storage stability of the product (32).

Table III Theoretical and Actual TMZ and Moisture Content of the Dry Powder Formulations (F1, F2, F3 and F4).

	Theoretical TMZ content	Actual TMZ content (Mean ± S.D., $n=3$)	Moisture content (Mean ± S.D., $n=3$)
F1	100%	100.5 ± 0.4%	0.3 ± 0.2%
F2	95%	96 ± 2%	0.24 ± 0.02%
F3	45.87%	77 ± 1%	0.42 ± 0.02%
F4	43.29%	70.6 ± 0.8%	0.58 ± 0.07%

The thermal properties determined by DSC (Fig. 2) confirmed the observations made by XRPD concerning the preservation of the initial TMZ crystalline structure. The exothermic peak temperature corresponding to the fusion-decomposition of raw TMZ was not modified for the F1 and F2 dry powder formulations. Nevertheless, this temperature was lowered to about 190°C, and the peak broadened for F3 and F4 due to the presence of relatively high amounts of DLPC, DMPC and α-lactose monohydrate in these formulations. The exothermic peak alteration for F3 and F4 can be attributed to the presence of relatively high amounts of these lower melting point excipients rather than an alteration of the crystalline nature of TMZ, because similar DSC curves were observed for the following physical mixtures: TMZ, DLPC and DMPC; TMZ and α-lactose monohydrate; and TMZ, DLPC, DMPC and α-lactose monohydrate (data not shown). In addition, an endothermic peak was observed at 139°C for F3 and F4 that corresponds to the dehydration of α-lactose monohydrate (35).

Particle Size and Aerodynamic Behavior of the Dried Formulations

Particle size was measured at different steps during the production of the formulations using a laser light-scattering technique (Table II). For each dried formulation, SEM was

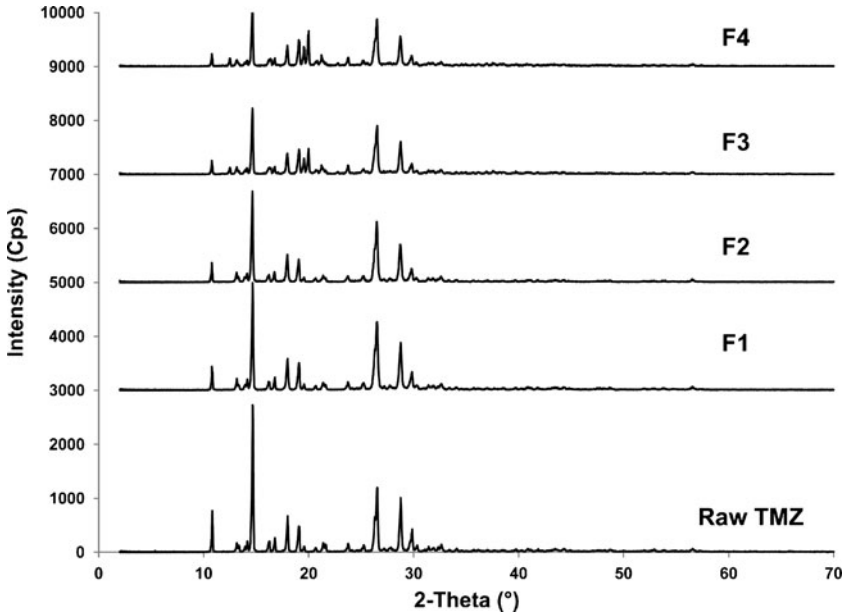


Fig. 1 X-Ray diffractograms of raw TMZ and the dry powder formulations.

used to visualize the morphology of the particles (Fig. 3 and 4), and the aerodynamic properties were evaluated using an MsLI (Table II and Fig. 5).

The size of the individual particles in each dried formulation increased in proportion to the amount of excipient added. The F1 particles (without excipient) exhibited the lowest size with a d(v,0.5) of 1.65 µm. In the F2 formulation, the presence of the lipid coating (4% of the TMZ weight) around the TMZ particles slightly increased the particle size to a d(v, 0.5) of 1.77 µm. Finally, the application of a lactose coating increased the particle size in the F3 (23% of the TMZ weight) and F4 formulations (29% of the TMZ weight) to a d(v,0.5) of 1.97 µm and a d(v, 0.5) of 2.75 µm, respectively.

Important factors involving how a powder deposits and how a drug is delivered to the lung include the following: the individualized particle size of the dried formulations (generally determined by laser diffraction from properly dispersed powders in an appropriate dispersion medium), the deagglomeration behavior in an air stream and the flowability. Laser light scattering provides the geometric diameters of individuated particles, and MsLI considers the agglomeration state of the dry powders under simulated breathing conditions. This allows aerodynamic diameter

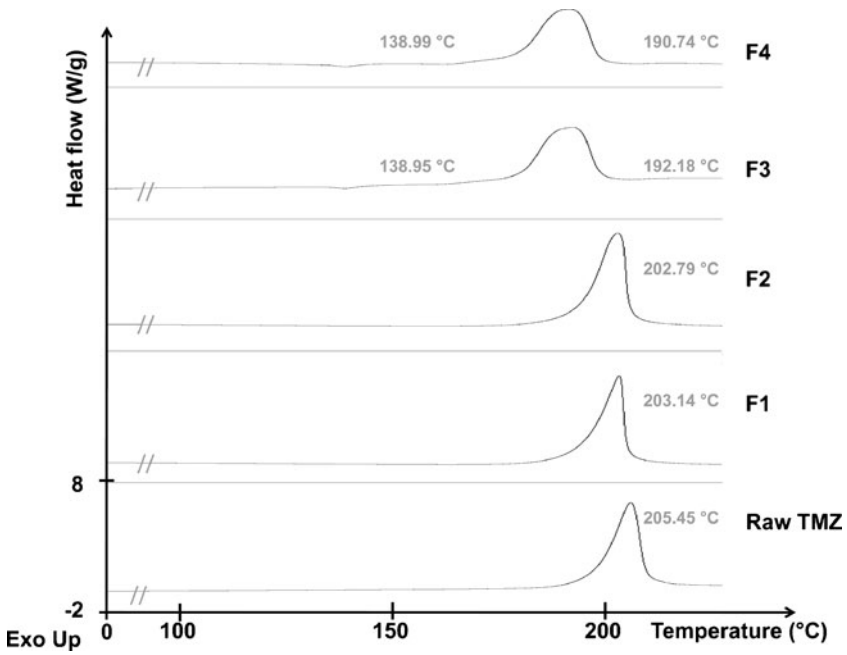


Fig. 2 DSC heating curves of raw TMZ and the dry powder formulations. The temperatures are noted for the endothermic peak of dehydration for α-lactose monohydrate (F3 and F4) and the exothermic peak of fusion-decomposition for TMZ (F1, F2, F3 and F4).

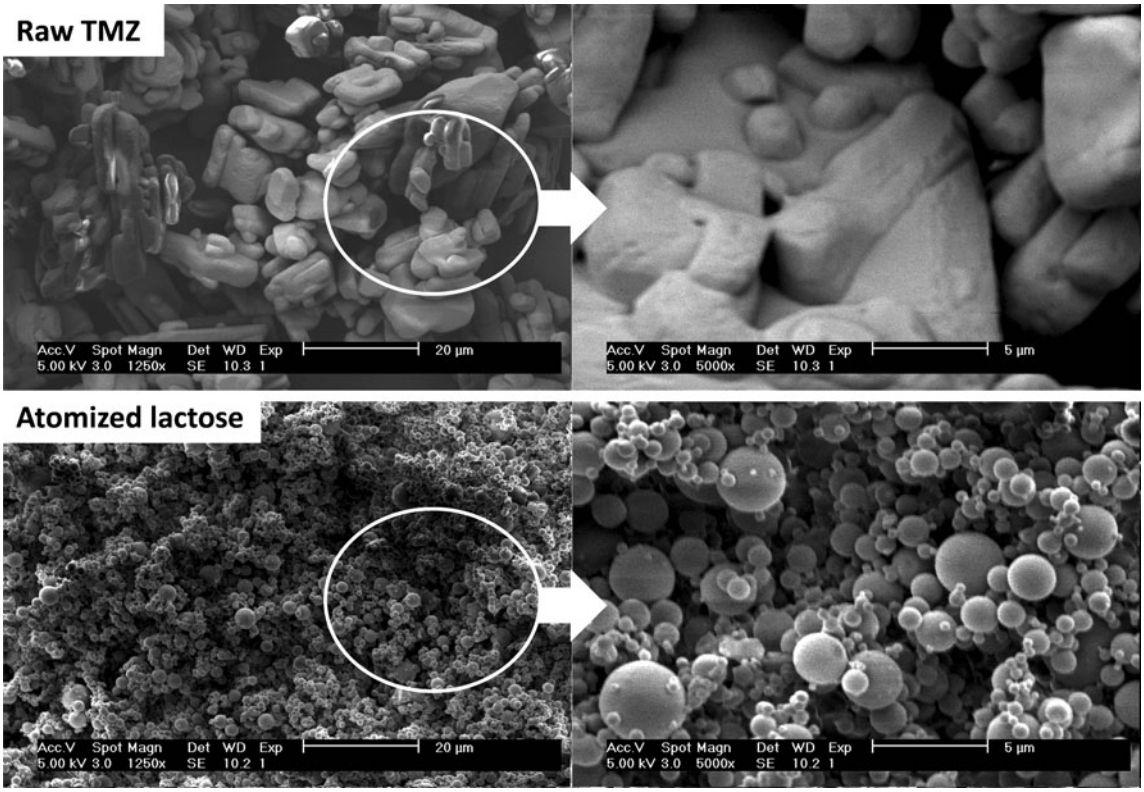


Fig. 3 SEM photographs of raw TMZ and lactose (spray-dried in the same conditions as formulations F3 and F4), at magnifications of 1250× and 5000×, respectively.

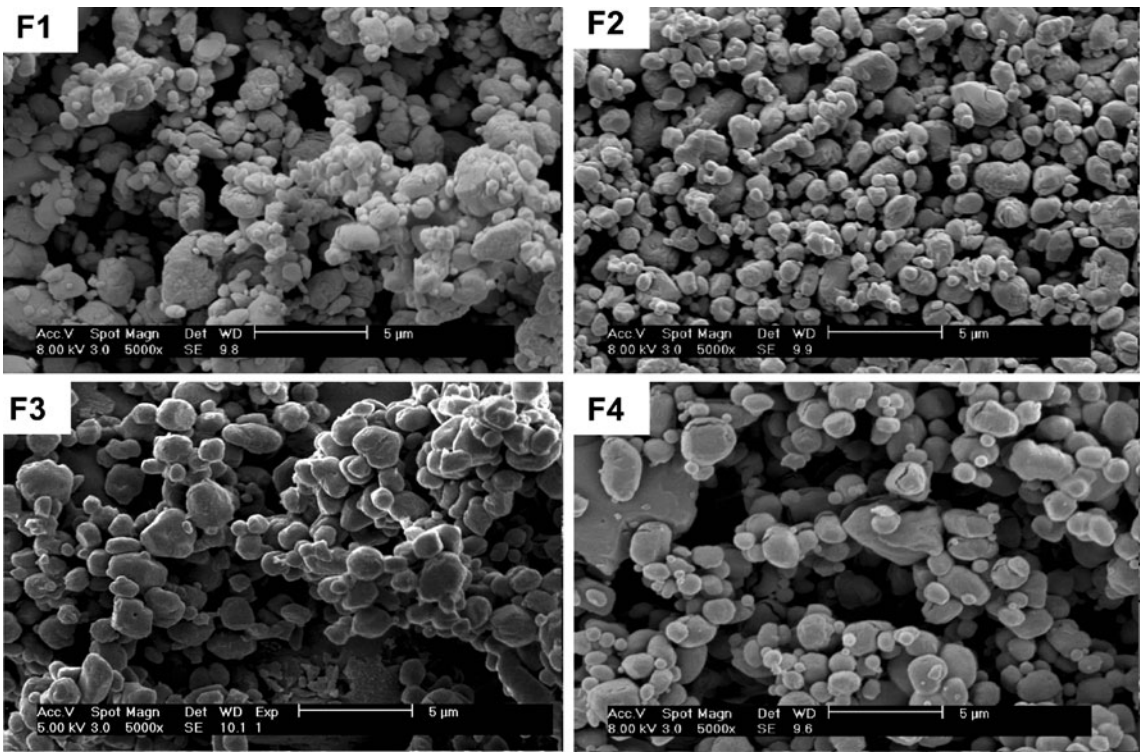


Fig. 4 SEM photographs of the dry powder formulations (F1, F2, F3 and F4) at a magnification of 5000×.

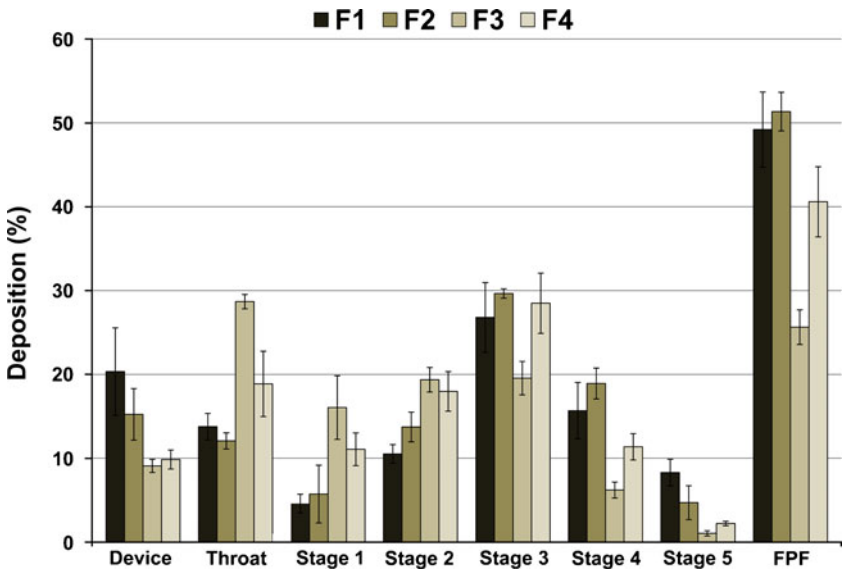


Fig. 5 *In vitro* deposition patterns and fine-particle fraction (FPF) of the dry powder formulations (F1, F2, F3 and F4) determined with an MsLI from the Axahaler® device (100 l/min, 2.4 s, and 3N° 3 HMPC capsules (20 mg/test)) (mean ± SD, $n = 3$).

measurements, which depend on the median geometric diameter, shape and density of the particle (36).

The morphology evaluation (Fig. 3 and 4) showed that the particles in the dried formulations were smaller and more spherical than the raw TMZ particles. The HPH process reduced the particle size and homogenized the particle shape. Moreover, the addition of lactose increased the size and sphericity of the particles (F3 and F4) in comparison to the excipient-free formulation F1. The SEM analysis also revealed that looser agglomerates were obtained for the F2 and F4 formulations, rather than the F1 and F3 formulations, which could be explained by the presence of a coating around the dried particles. These surface modifications, due to the presence of a lipid or lactose coating, could influence the dispersion of the TMZ particles by decreasing interparticle interactions and, consequently, their deposition in the lungs, as has been previously shown for tobramycin (33).

The aerodynamic behavior was characterized by MMAD (μm), FPF (%) and FPD (mg) (Table II). The TMZ recoveries from the inhalator to the filter of the MsLI were between 79 and 95% of the total loaded drug. Moreover, the MMAD for F4 was lower than F3, although laser light-scattering analysis showed a higher particle size for F4 than F3. This could be explained by a decrease in the density and interparticle interactions due to the presence of a thicker lactose coating. In fact, F4 contained a higher quantity of lactose than F3, which could more efficiently decrease the dry particles that stick together due to the presence of phospholipids that possess low melting ($T_{melting}$) temperatures.

The deposition patterns for the different dried inhalation formulations at different stages in the MsLI are presented in Fig. 5. The F1 and F2 dried formulations presented the best aerodynamic characteristics with minimal deposition in the induction port, stage 1 and stage 2, which simulated deposition in the throat and trachea, respectively. These formulations also showed the highest deposition in stages 3, 4 and 5, which simulated deposition in the conducting and respiratory airways zones. Moreover, F2 seemed to show a slightly higher FPF with less variability than F1 in the deposition profile, which could be explained by the presence of the lipid coating. It has been shown that the lipid coating for a hydroscopic drug, such as tobramycin, drastically improved aerodynamic performance by limiting absorption of the ubiquitous vapor and thus reducing agglomeration tendency (33). Because TMZ does not show any hygroscopic character, no significant increase was shown in terms of the deposition profile for F2 in comparison to F1. In contrast, the F3 and F4 dried formulations presented worse deposition profiles than F1 and F2, probably because of the relatively high amounts of lower melting phospholipids in F3 and F4 (DLPC: $T_{melting} \sim 47°C$ and DMPC: $T_{melting} \sim 49°C$) relative to F2 (P90H; $T_{melting} \sim 120°C$) and F1 (without excipient). DLPC and DMPC were necessary to obtain a homogeneous TMZ dispersion in an aqueous medium during the particle size reduction step by HPH. These lower melting phospholipids could promote the dry powder particles sticking together so an increasing amount of lactose has been added before the spray-drying step. Lactose influenced the aerodynamic performance, and this is shown by the better deposition results observed for F4 (higher lactose content) than for F3; however, both formulations still remained lower than F1 and F2.

It is important to keep in mind that primary lung cancers or pulmonary metastases can invade the conducting zone of the airways (extending from the trachea to the terminal

bronchioles) and the respiratory zone (including the respiratory bronchioles, alveolar ducts and alveolar sacs) (16,17). Kleinstreuer and Zhang (16) evaluated a lung airway model with bronchial hemispherical tumors to analyze drug aerosol deposition in airflow conditions expected in patients with tumors in the conducting zone. Their aim was to validate the concept of "controlled particle release and targeting" by maximizing deposition on the tumor surface and minimizing deposition on nearby healthy tissue. This was achieved by controlling the air-particle stream that was generated by a specific inhaler with knowledge of lung morphology, the afflicted lung area, the breathing mode and the drug aerosol characteristics. In a non-controlled air-particle stream, the particle deposition occurred mainly along the front surface of the tumor due to impaction. The presence of small-to-medium-size tumors (e.g., a ratio of tumor radius to local airway radius, in the range of 0–1.25) resulted in a reduction in the flow rate and an increase in the particle deposition with tumor growth due to inertial impaction. However, in the case of large-size tumors (e.g., a ratio of tumor radius to local airway radius, in the range of 1.25–2), the flow rate decreased drastically in the bifurcation where the tumor was localized, and particle deposition was low. Consequently, the pulmonary delivery of chemotherapeutic aerosols, the particle size distributions and the breathing parameters need to be specifically engineered and controlled to optimize these conditions.

Release Profile of TMZ from Dry Powder Formulations

After the deposition of drug particles in the lungs, the drug has to dissolve to be available to cancer cells before being eliminated by the clearance systems. These systems are the mucociliary escalator in the conducting zone, and macrophages (via phagocytosis) and systemic absorption after dissolution in the respiratory zone. In a previous study, we showed that, to obtain an equivalent antitumor efficacy by inhalation, we had to deliver the same TMZ dose at a similar frequency to that used for the intravenous route (25). These high doses and frequencies could be explained by the rapid dissolution and elimination of the liquid suspension with the TMZ particles from the lung. However, using dry powders for the inhalation route could decrease these parameters because a powder is dissolved more slowly than particulates in a liquid suspension. It is known that the mucociliary clearance rate of a normal person's lung is about 1–2% per min (half-life is about 1–2 h) (37). The mucociliary particle clearance is not influenced by the particle size, as demonstrated for a polystyrene particle of 50 nm to 6,000 nm, but it seems to be influenced by the surface chemistry of particle (38). The smaller particles (below 3 μm) are mainly deposited deep into the respiratory zone, where the alveolar macrophages and systemic absorption are the main clearance systems. Clearance by macrophages is significantly slower than mucociliary clearance. Insoluble particles in the alveoli can thus reside for days before being completely removed by phagocytosis, which depends on the particle size, shape and load (37).

Dissolution is the process by which a solid substance enters into a solvent to yield a solution and is controlled by the affinity between the solid substance and the solvent (37). For some drugs, such as nifedipine (30) or itraconazole (39), the dissolution rate can be a limiting factor for their efficacy. TMZ is slightly water-soluble, and some improvements need to be performed to overcome this problem (40). There are favorable conditions in the lungs that promote TMZ dissolution, such as a larger deposition area and the presence of lung surfactant, but there is also a limitation on dissolution due to the small fluid volume (~100 ml). Standardized dissolution test methods are used for various pharmaceutical dosage forms to predict the dissolution rate and, consequently, the *in vivo* dissolution behavior of the drug. However, to date, no pharmacopeial method exists to determine the *in vitro* dissolution rate for inhaled products. Davies and Feddah (37), Salama *et al.* (41) and Son and McConville (42) have suggested several methods, but none have been adopted.

In this study, we used a recent *in vitro* dissolution test method that was optimized for inhalation formulations and described by Son *et al.* (DDL Poster 2009). As recommended, an aerodynamic selection was made to limit the variation due to the particle size distribution on the dissolution profile. This selection was also made to determine the release profile on the fraction of dry powder that possessed the higher deposition level with an aerodynamic diameter less than 5 μm. Therefore, stage 3 was chosen with a percentage of deposition related to the TMZ metered dose that was ~15–20% for F1 and F2 and ~10–15% for F3 and F4. These particles displayed aerodynamic diameters between 2.82 and 4.46 μm.

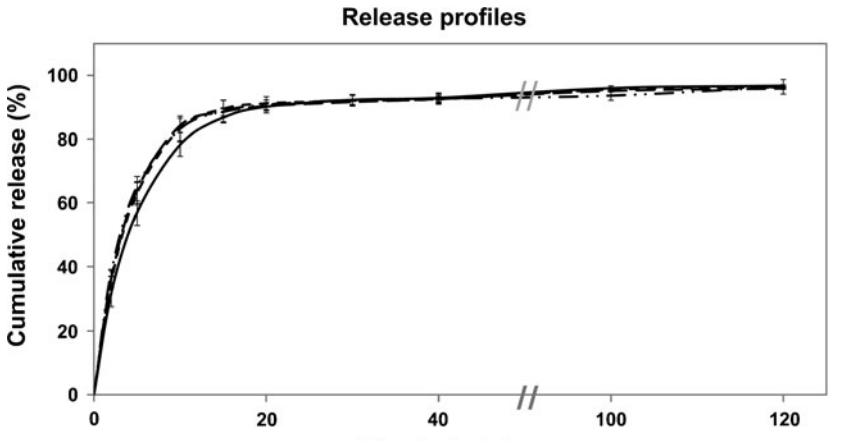
The dose was collected on the dose collection body, a polycarbonate membrane was placed on top of it and the cassette was sealed. In such a system, some air can be trapped under the membrane and slow down the contact between the dissolution media and the dry powder. Consequently, the concentration determined at 180 min was taken as that for 100% TMZ dissolution to minimize the variation due to this area of trapped air. One actuation was made to obtain well-dispersed particles in approximately a single layer. The dissolution medium used was SLF fixed at pH 5.0 instead of pH 7.4 to guarantee the stability of TMZ during the test. It is important to note that the solubility of TMZ (~3 mg/ml at 25°C) is independent of pH because TMZ has no ionizable function. The stability of TMZ is decreased above pH 6, where the

prodrug TMZ was hydrolysed to 5-(3-methyltriazen-1-yl) imidazole-4-carboxamide (MTIC) (0.8%, 12% and 66% of TMZ was hydrolyzed after 3 h at 37°C at pH 5.0, 6.0 and 7.4, respectively).

The dissolution profiles of TMZ for all the dry powder formulations (Fig. 6) were similar to the profile obtained from the F1 formulation. This is shown by the similarity factor f_2, which was higher than 50 (f_2=76,74 and 71 for F2, F3 and F4, respectively). This similarity in the dissolution profiles can be explained by the small particle sizes and the porous nature of the coating, as it was obtained by a very rapid drying step from a solution of lipids or lactose during the spray-drying process. For these reasons, the coating was not able to slow down the TMZ release from the formulations in comparison to F1, which does not have any surface coating. Moreover, the dissolution profile for F1 was comparatively lower than those obtained from the coated formulations for the first 15 min. The coating containing a hydrophilic compound, such as lactose (F3 and F4), or surfactant, such as phospholipids (F2, F3 and F4), could promote the dispersion of the particles, however, not enough to observe significant differences. The profiles reveal that more than 75% of the TMZ was released within 10 min. Moreover, the presence or absence of 0.2% DPPC in the composition of the SLF did not change the release profile of TMZ from dry powder F1 (f_2=68), which suggests that TMZ presents no problem of wettability. The excipients added in F2, F3 or F4 did not affect the release of TMZ in the experimental conditions adopted *in vitro*, and this slightly water-soluble drug should probably be dissolved without any difficulties in the lung. In the future, *in vivo* experimentations should be performed to evaluate the efficacy of dry powders and their possible impact on decreasing the dose and frequency of TMZ delivery by inhalation.

CONCLUSIONS

This study demonstrated that it is possible to produce TMZ-based dry powder formulations with high TMZ content for inhalation. The unchanged crystalline state of TMZ in these formulations and their low moisture content promote the long-term stability of the formulations. A fast drug release was observed for all dry powder formulations, and more than 75% of the TMZ was released after 10 min. TMZ is considered a slightly water-soluble drug but presented no problem dissolving in the SLF for particles displaying aerodynamic diameters suitable for the inhaled route. Good aerodynamic profiles were observed for the different formulation approaches used with better results for formulations without excipient (F1) or with a lipid coating (F2). Moreover, F2 showed a slightly higher FPF with less variability in comparison with F1. Finally, F1 and F2 displayed the highest TMZ content and the lowest moisture content. Consequently, the TMZ-based dry powder formulations for inhalation without or with a lipid coating seem to be the most promising for targeting pulmonary tumors. In the future, the *in vivo* activity of the dry powders for inhalation should be evaluated. Based on these results, dry powder formulations with controlled-release properties, which escape mucociliary clearance and alveolar macrophages, could then be developed to optimize inhalation treatment delivery.



	2	5	10	15	20	30	40	100	120
F1	31 ± 4	57 ± 4	78 ± 4	87 ± 2	90 ± 1	92 ± 1	93 ± 1	96 ± 1	96.8 ± 0.5
F2	35 ± 2	63 ± 4	83 ± 4	90 ± 3	91 ± 2	92 ± 2	92 ± 1	95.6 ± 0.8	96.4 ± 0.3
F3	36 ± 3	64 ± 4	84 ± 2	89 ± 1	90 ± 1	91.7 ± 0.8	92.6 ± 0.8	95.2 ± 0.3	95.9 ± 0.3
F4	38 ± 1	65 ± 1	83 ± 4	89 ± 3	91 ± 2	92 ± 2	93 ± 2	94 ± 2	96 ± 2

Fig. 6 Release profiles of TMZ from the dry powder formulations for inhalation (F1, F2, F3 and F4; mean ± S.D., n=3). These profiles were determined after impaction (NGI with an Axahaler® device at 60 l/min, 4 s, and 1 N°3 HPMC capsule) of 5 mg of particles (aerodynamic diameter: 2.82 to 4.46 μm) on the membrane cassette placed into a vessel of the USP 33 type II dissolution apparatus (300 ml of SLF at pH 5.0, 37°C, paddle operating speed of 75 rpm).

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