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 means of a high-pressure homogenizing technique and the solvent was evaporated by spray-drying to obtain a dry powder. Physicochemical properties (crystalline state, thermal properties, morphology, particle size and moisture and drug content) were determined by means of X-ray powder diffraction, differential scanning calorimetry, scanning electron microscopy, laser light scattering, thermogravimetric analysis and high-performance liquid chromatography, respectively. Aerodynamic properties and release profiles were also evaluated using a multistage liquid impinger and a modified USP type 2 dissolution apparatus adapted to inhaler products, respectively.

Results The dry powder formulations for inhalation had a high temozolomide content that ranged from 70 % to 100%, remains the temozolomide crystalline state and had a 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 pulmonary delivery, dry powder, lung cancer, inhalation chemotherapy, temozolomide
ABBREVIATIONS

NSCLC  non-small-cell lung cancer
SCLC  small-cell lung cancer
DPI  dry powder inhaler
TMZ  temozolomide
DLPC  1,2-dilauroyl-sn-glycero-3-phosphocholine
DMPC  1,2-dimyristoyl-sn-glycero-3-phosphocholine
P90H  phospholipon 90H
HPH  high-pressure homogenizing
XRPD  X-ray powder diffraction
SEM  scanning electron microscopy
DSC  differential scanning calorimetry
TGA  thermogravimetric analysis
HPLC  high-performance liquid chromatography
MsLI  multi-stage liquid impinger
FPD  fine particle dose
FPF  fine particle fraction
HPMC  hypromellose
NGI  next generation impactor

MMAD  mass median aerodynamic diameter

MTIC  5-(3-methyltriazen-1-yl)imidazole-4-carboxamide

SLF  simulated lung fluid

DPPC  dipalmitoyl phosphatidylcholine
INTRODUCTION

Lung cancer remains 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 of metastatic processes from prostate, breast, colorectal, kidney, head and neck carcinomas, and also from sarcomas and melanomas (3,4). 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 in order to reduce 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 chemotherapy is delivered by infusion via a parenteral route and causes significant systemic toxicities to the patient. These toxicity-related features imply interruption of the treatment to allow normal tissue to recover, a process that occurs in parallel with tumor cell repopulation in various organs (5). In addition, because of this dose-limiting toxicity, there is only a modest increase in patient survival time, probably because effective therapeutic concentrations of the cytotoxic drugs of interest are not reached at the tumor site via the infusion route (5,6). Moreover, chronic IV treatments of cancer patients, including NSCLC patients, are associated with multiple adverse events including damaged veins, infection at the site of catheter introduction or air embolisms via the intravenous line (7).

Delivery of chemotherapeutic agents, including cytotoxic drugs, via the pulmonary route to treat lung tumors has been investigated since 1968 (8). As reviewed by Gagnadoux et al. (9) and Smyth et al. (5), an increasing number of preclinical studies and early clinical trials demonstrate the clinical potential and feasibility of this approach, which enables a high therapeutic ratio to be achieved along with sharp decreases in severe systemic side effects.
The most adverse events observed when treating lung cancer patients through the inhalation route are related to the direct toxic effects of the inhaled drug on the upper and lower respiratory tract and depend on the dose and the drug that is administered. In most of the reports available from the literature, air jet nebulizers are used as the inhalation device for cytotoxic chemotherapeutics (10,11,12). However, these devices display many disadvantages including being cumbersome and requiring additional tubing and mouth pieces in addition to compressed air and/or oxygen sources. Moreover, they present extended administration time, high cost, risk of device contamination and face and environmental exposure. These devices display, in general, low efficiency and poor delivery reproducibility and require regular maintenance (13).

Another approach to delivering cytotoxic chemotherapeutics to the lung could be the 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 (DPI) present many advantages when compared to liquid nebulizer systems because DPI-based formulations are in solid state, which is more stable for long-term storage and better adapted to poorly water soluble drugs such as conventional cytotoxic chemotherapeutics. 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 and residence of therapeutic aerosols to and in the desired regions of the airways is directly dependent upon a combination of aerodynamic and physicochemical characteristics of the inhaled particles, patient inhalation dynamics and lung physiology/disease (5).

In this study, we have produced and evaluated TMZ-based dry powder formulations without and with excipients to deliver a high drug dose to the pulmonary tract. The excipients chosen were lactose, phospholipids and cholesterol, all of which are well-tolerated by the respiratory
tract (14). These excipients could influence the aerodynamic characteristics and the
dissolution profile of the powders in the lungs. Moreover, the aerodynamic characteristics
were optimized to deliver chemotherapeutic drugs to tumors that could be located in the
conducting zone as well as in the respiratory zone of the lungs (15,16).

We have chosen TMZ as the model drug because it is clinically active against cancers
associated with extremely poor prognoses, such as glioblastomas and melanomas, as well as
experimental cancer models, including pre-clinical models of NSCLC, breast, prostate,
ovidian, and head and neck cancers (17). In addition, TMZ, as a cytotoxic cancer-treatment
agent, presents an acceptable safety profile (18). In fact, TMZ is an alkylating agent that
induces sustained pro-autophagic effects in cancer cells, a feature that in turn leads to
apoptosis in these cancer cells (17). TMZ has also recently been assayed in a handful of
clinical studies (Phase I and II) for NSCLC patients (19,20,21, http://www.clinicaltrials.gov
website). The fact that TMZ induces sustained pro-autophagic effects in cancer cells, with an
ultimate consequence (but not a direct cause) of apoptotic cell death, could overcome the
intrinsic resistance of a number of cancer types (NSCLCs, glioblastomas, melanomas,
pancreas cancers and esophageal cancers, among others) to cytotoxic drugs that induce pro-
apoptotic effects as a direct effect of their mechanism of anticancer action (17,22). We
recently demonstrated the in vivo therapeutic benefits contributed by inhaled TMZ in a mouse
melanoma pulmonary pseudometastatic model (23) that displays significant resistance to pro-
apoptotic stimuli (24). The current study aims to develop dry powder formulations usable in
humans with good physicochemical properties ensuring long term stability, interesting
aerodynamic behaviors and no problems with dissolution in the lung.
MATERIALS AND METHODS

1. Materials

Temozolomide (TMZ) was supplied from Shilpa Medicare Limited (Raichur, India); 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) and phospholipon 90H (P90H), hydrogenated soy lecithin, with more than 90% hydrogenated phosphatidylcholine and 15% dipalmitoyl phosphatidylcholine, was donated by Nattermann Phospholipids (Koln, Germany). Dipalmitoyl phosphatidylcholine (DPPC) was purchased from Lipoid (Ludwigshafen, Germany). 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.

2. Methods

2.1. Hazardous drug procedures

TMZ is a hazardous drug and procedures were therefore elaborated to protect the manipulator and the environment. Personal protective equipment was composed of 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. The respirator used was a FFP3 particle-filtering face piece (3M, Cergy-Pontoise, France). Eye glasses 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) designed for our application with the air circulated through high-efficiency particulate air (HEPA) filters before being eliminated outside of the building.

2.2. Preparation of the TMZ dry powder formulations – high pressure homogenizing (HPH) and spray drying

The theoretical composition of the suspensions that were used to prepare dry powders by spray-drying is described in Table I. TMZ in F1 and F2 was dispersed using a high-speed stirrer-homogenizer composed of an X620 motor coupled with a T10 dispersing tool (CAT M. Zipperer, Staufen, Germany). The procedure involved a homogenizing time of 10 min at a homogenizing speed of 24,000 rpm in an ice bath to prevent sample temperature increase.

TMZ in F3 and F4 was dispersed in a dispersion of DLPC and DMPC, which were previously mixed in phosphate buffer (pH 5.0), using the same procedure as described for F1 and F2.

The size-reduction step was performed by means of an EmulsiFlex-C5 high-pressure homogenizer (Avestin Inc., Ottawa, Canada). Pre-milling low-pressure homogenizing cycles (15 cycles at 4,000 PSI and 10 cycles at 12,000 PSI) were performed with the TMZ suspensions (to avoid blocking the homogenizing gap) before applying 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 the suspension. All experiments were performed using a heat exchanger downstream of the homogenizing valve in order to maintain a relatively low and constant temperature of 2.5 ± 1 °C for suspensions with isopropanol and 10 ± 1 °C for suspensions in phosphate buffer in order to limit evaporation and heating of the suspensions during the process. P90H and cholesterol for F2 and lactose for F3 and F4 were dissolved by stirring after having determined the TMZ content by high-pressure liquid chromatography.
(HPLC). Finally, these suspensions were spray-dried using a B-290 Mini Spray Dryer (Büchi Laboratory-Techniques, Flawil, Switzerland) at fixed relative humidity (50-60%) by means of a B-296 dehumidifier (Büchi Laboratory-Techniques, Flawil, Switzerland). The suspensions were kept stirring and 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 was at a rate of 35 m³/h and was heated to 70 °C for F1 and F2 and 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 acyclone separator and were collected in a container. The process yield was about 40% for F1, 60% for F2 and 50% for F3 and F4 with a volume of 50 ml of isopropanolic suspension and 90 ml of aqueous suspension spray-dried following the conditions described above. The dried formulations were stored in a desiccator at ambient temperature.

2.3. Particle size – laser light scattering

The particle size distribution of raw TMZ, of the dry powder formulations after redispersion in isopropanol for F1, F3 and F4 and in water for F2 and of the corresponding suspensions after the size-reduction step were measured by means of a Malvern Mastersizer 2000 laser diffractometer using a Hydro 2000 wet sampling system (Malvern Instruments Ltd, Worcestershire, UK). 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.

2.4. 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 lactose spray-dried in 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 at 35 mA for 90 sec at 5.10^{-2} mbar under argon.

2.5. 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 2θ range of 2θ-70 °.

2.6. 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 to be
analyzed ranged from 1-3 mg and was placed in Tzero aluminum pans. Heat runs for each
sample were set from 0 °C to 230 °C at 5 °C/min using nitrogen as a blanket gas.

2.7. Moisture content determination – thermogravimetric analysis (TGA)
The amount of residual water in raw TMZ and the dried formulations was assessed by TGA by means of 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. Samples weighed approximately 10 mg. The moisture level was determined by the weight loss obtained between 25 °C and 125 °C.

2.8. TMZ determination – high-performance liquid chromatography (HPLC)

The determination of TMZ in the dry powder formulations, in the aerodynamic particle size analysis and in the determination of 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% v/v aqueous acetic acid-acetonitrile (90:10 v/v). The volume injected was 10 μl, the temperature was set at 25 °C and the analysis time was 10 min.

2.9. Aerodynamic particle size analysis – multi-stage liquid impinger (MsLI)

The fine particle dose (FPD) and aerodynamic particle size distribution were determined by following the procedure for powder inhalers using Apparatus C, i.e. 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 test of uniformity of delivered dose for obtaining a pressure drop across the inhaler of 4 kPa, i.e., 100 l/min. The cut-off diameters at this test flow rate for the MsLI were 5.27, 2.40 and 1.32 \( \mu m \) 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 liters of air from the mouthpiece of the inhaler and through the MsLI at the test flow rate, i.e., 2.4 sec. 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 was 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. The FPD is defined as the mass of active substance with aerodynamic diameters smaller than 5 \( \mu m \). 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 and 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, whereas the emitted dose is defined as the total powder mass exiting the capsule and device.

2.10. 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). A membrane cassette containing a certain
dose of TMZ and consisting of a polycarbonate membrane (0.1 μm diameter pore) (Copley
Scientific Limited, Nottingham, UK) and a stainless steel membrane holder (Copley Scientific
Limited, Nottingham, UK), composed of a quick release dose plate, a dose collection body
and a sealing ring, was placed in the bottom of the dissolution vessel.

Collection of the dose into the membrane cassette was conducted using a Next Generation
Impactor (NGI) (Copley Scientific Limited, Nottingham, UK) with the dose collection body
fixed at the quick release dose plate at stage 3. The DPI device used was an Axahaler® (SMB,
Brussels, Belgium) containing a N°3 HPMC capsule (Capsugel®, Colmar, France) filled with
an appropriate dose of each formulation for obtaining about 5 mg of TMZ at stage 3 after
dispersal into the NGI through the appropriate induction port at a flow rate of 60 l/min for 4
sec. 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 a dissolution vessel.

The dissolution conditions were (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, composed of simulated lung fluid (SLF) as described by Sdraulig
et al. (252008) 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.

2.11. Statistical analyses
The similarity of dissolution profiles was determined using the similarity factor $f_2$, as recommended by the FDA’s Guidance for Industry. The similarity factor $f_2$ value must be higher than 50 to assess the similarity between two dissolution profiles (26).
RESULTS AND DISCUSSION

The approved conventional treatment regimen of TMZ for recurrent gliomas is a daily dose of 150-200 mg/m² of body-surface area by infusion or by mouth for 5 days, repeated every 28 days (27). In our previous in vivo experimental study relying on the use of a mouse melanoma pseudometastatic lung model (24), we obtained the same efficacy in terms of median survival period when comparing TMZ administered through inhalation to TMZ administered intravenously (23). In addition, the local inhaled therapy resulted in long-term mouse survivors who showed an almost complete eradication of lung tumors (23). Considering a potential transposition of such a treatment in humans, if no reduction in the dose is 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, so the development of formulations that minimize the amount of excipient and that optimize the aerodynamic and dissolution properties of the inhalation powder is an important issue. With this aim, different formulations of dry powders for inhalation with no excipient or with a lipid or lactose coating were developed with the aim of optimizing these parameters and reducing the administered dose by inhalation.

3.1. 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, with 99.6% of particles displaying a size spread of 1-5 μm, as compared to only 5.0% before the size reduction process (Table II). After the spray-drying of the suspension and evaporation of the solvent (isopropanol for F1 and F2 and water for F3 and F4), TMZ content was determined for each dry powder formulation (Table III). The actual TMZ content of F1 and F2 corresponds to what was expected in “theory” according to the amount of drug introduced in the initial suspensions (i.e., the theoretical TMZ content). However, F3 and F4 showed a higher TMZ content than the theoretical composition. It thus seems that the lactose coating
crumbled away during the drying process. Consequently, the actual TMZ content is the value that has to be considered in the *in vitro* and the future *in vivo* evaluations.

3.2. Physicochemical characterization of dried formulations

XRPD patterns (Fig. 2) 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 the drug after HPH and spray-drying processing have already been demonstrated for other drugs such as nifedipine (28) and tobramycin (29). Each diffraction peak observed for the dry powder formulations corresponds to those obtained for the raw TMZ. The F3 and F4 formulations had additional peaks at 12.5, 16.4, 20.0 and 20.9 °, corresponding to α-lactose monohydrate (30). No diffraction peaks characterizing cholesterol and P90H were observed for F2. This could be explained by the lack of sensitivity of the method and the limited coating level for the lipid coated formulation (5% of TMZ weight) (31). The moisture content, as evaluated by TGA, was very low, below 1% (Table III). 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 residues of solvents that could be found are, in this case, water and isopropanol. 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 (32); this is generally determined by gas chromatography.

The presence of the initial crystalline form and the low moisture content are both important in order to guarantee the long-term storage stability of the product (30).

The thermal properties determined by DSC (Fig. 3) confirmed the observations made by XRPD concerning the preservation of the initial crystalline state of TMZ. The temperature of the exothermic peak corresponding to the fusion-decomposition of the raw TMZ was not modified for the F1 and F2 dry powder formulations. Nevertheless, this temperature was
lowered to about 190 °C for F3 and F4 due to the presence of α-lactose monohydrate, which recrystallizes (exothermic peak) at 173 °C (33). Moreover, for F3 and F4, an endothermic peak was observed at 140 °C that corresponds to the dehydration of α-lactose monohydrate (33).

### 3.3. Particle size and aerodynamic behavior of the dried formulations

Particle size was measured at different steps of the production of the formulations using a laser light scattering technique (Table II). For each dried formulation, the morphology of the particle was visualized by SEM (Fig. 4 and 5) and the aerodynamic properties were evaluated using an MsLI (Table II and Fig. 6).

The size of the individualized particles in each dried formulation increased in proportion to the amount of excipient added. Indeed, particles of F1 (without excipient) exhibited the lowest size with a d(v,0.5) of 1.65 μm. The presence of the lipid coating around TMZ particles in F2 slightly increased the particle size to obtain a d(v, 0.5) of 1.77 μm. Finally, the application of lactose coating increased the particle size of F3 (d(v,0.5) of 1.97 μm) and, to a greater extent, the size of F4 particles, which had a d(v, 0.5) of 2.75 μm.

Besides the individualized particle size of the dried formulations, the deagglomeration behavior in an air stream as well as the flowability are important factors in how a powder deposits in the lungs and how drug delivery to the lung might occur. Laser light scattering gives the geometric diameters of individuated particles, whereas MsLI considers the agglomeration state of the dry powders under simulated breathing conditions and allows the measuring of the aerodynamic diameter, which depends on the median geometric diameter, shape and density of the particle (34).

The morphology evaluation (Fig. 4 and 5) showed that the particles in the dried formulations were smaller and more spherical than the particles of raw TMZ. Indeed, the HPH process
reduced the particle size and homogenized the particle shape. Moreover, the addition of lactose in solution 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 F2, F3 and F4 give particles that are less cohesive than F1, which could be explained by the presence of a coating around the dried particles. These surface modifications could influence the dispersion of the TMZ particles (by decreasing interparticle interactions) and consequently, their deposition in the lungs (31).

The deposition pattern of the different dried formulations for inhalation at different stages of the MsLI is presented in Fig. 6. The aerodynamic behavior was characterized by the mass median aerodynamic diameter (MMAD) (μm), FPF (%) and FPD (mg) (Table II). The TMZ recoveries from the inhalator up to the filter of the MsLI were in the range of 79%-95% of the total loaded drug. Moreover, the MMAD from F4 was lower than from F3, whereas laser light scattering analysis showed a higher particle size for F4 than F3. That could be explained by a decrease of the density and interparticle interactions brought about by the presence of a thicker lactose coating; F4 particles showed a smaller aerodynamic diameter but a bigger geometric diameter than F3 particles.

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, and highest deposition in stages 3, 4 and 5, which simulated the deposition in the conducting zone and respiratory zone of the airways.

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) as well as the respiratory zone (including the respiratory bronchioles, alveolar ducts and alveolar sacs) (15,16). Kleinstreuer and Zhang (15) elaborated a lung airway model with bronchial
hemispherical tumors in order to analyze the drug aerosol deposition in airflow conditions expected in patients bearing 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 could be generated by a specific inhaler with knowledge of the lung morphology, the afflicted lung area, the breathing mode and the drug aerosol characteristics. It was shown that 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 increasing 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 chemotherapy aerosols, the particle size distributions and the breathing parameters need to be specifically engineered and controlled to optimize these conditions.

3.4. Release profile of TMZ from dry powder formulations

After the deposition of particles in the lungs, the drug has to dissolve to be available to cancer cells before being eliminated by the clearance systems, which 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 those used for the intravenous route (23). These high doses and frequency could be explained by fast dissolution and elimination of the liquid suspension containing the TMZ particles from the lung. Dry powders for inhalation could decrease these parameters because a
powder is dissolved more slowly than particles pre-dispersed in a liquid saturated in drug such as a suspension. It is known that the mucociliary clearance rate of a normal person’s lungs is about 1-2% per min, i.e., half-life is about 1-2 h (35). The clearance by macrophages is significantly slower compared with mucociliary clearance. Insoluble particles in the alveoli can, therefore, reside for days before being completely removed by phagocytosis, depending on particle size, shape and load (35).

Dissolution is defined as 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 (35). For some drugs, the dissolution rate can be a limiting factor for their efficacy, i.e., nifedipine (28) or itraconazole (36). TMZ is a compound that is very slightly soluble in water and some strategies are performed in order to overcome this problem (37). In the lungs, there are some favorable conditions that promote TMZ dissolution, such as a huge deposition area and the presence of lung surfactant, but there is also a limitation on dissolution regarding the small fluid volume, i.e., ~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 pharmacopeia method exists to determine the in vitro dissolution rate from inhaled products. Some methods have been suggested by Davies and Feddah (35), Salama et al. (38) and Son and McConville (39), but none have been accepted yet.

In this study, we used a recent in vitro dissolution test method optimized for inhalation formulations described by Son et al. (DDL Poster 2009). As recommended, an aerodynamic selection was made in order to limit the variation due to the distribution of particle size on the dissolution profile and to determine the release profile on the fraction of dry powder that possesses 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 an aerodynamic diameters spread of 2.82-4.46 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 to be that for 100% TMZ dissolution to minimize the variation due to this area of trapped air. One actuation was made in order to obtain well-dispersed particles in approximately a single layer. The dissolution media used was SLF fixed at pH 5.0 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 below pH 6, where the alkaline hydrolysis converted TMZ 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. 7) were similar to the profile obtained from the F1 formulation, as revealed by the similarity factor f2 which was higher than 50. The profiles reveal that more than 75% of the TMZ was released after 10 min. Moreover, the presence or absence in the composition of 0.2% DPPC in the SLF did not change the release profile of TMZ from dry powder F1 (data not shown), which suggests that TMZ presents no problem of wettability. The excipients added in F2, F3 or F4 did not affect the release of TMZ, and this very slightly soluble in water drug should be dissolved long before being eliminated by the different clearance systems in the lung. In the future, in vivo experimentations should be performed to evaluate the efficacy of dry powders and their possible impact on the decreasing of dose and frequency of TMZ delivery by inhalation.
CONCLUSIONS

This study demonstrated that it is possible to produce TMZ-based dry powder formulations for inhalation with a high TMZ content. The unchanged crystalline state of TMZ in dry powder formulations and their low moisture content ensure the long-term stability of the formulations. A fast drug release was observed for all dry powder formulations, with more than 75% TMZ released after 10 minutes. TMZ is considered a very slightly soluble in water drug, but presented no problem of dissolution in the SLF for particles displaying aerodynamic diameters suitable for the inhaled route. Good aerodynamic profiles were observed for the different formulation approaches used. The dry powder formulations for inhalation without or with a lipid or lactose coating seem to be promising for targeting pulmonary tumors. In the future, the in vivo activity of the dry powders should be evaluated and, according to the obtained results, dry powders formulation with controlled-release properties could be developed in order to increase the efficacy of the treatments delivered by inhalation. Therefore, these dry powder formulations for inhalation could be administered in patients bearing pulmonary tumors after they have fulfilled the regulatory requirements for clinical studies, which could be facilitated by the use of physiologic and/or accepted excipients for this delivery route. Currently, TMZ is undergoing a large number of clinical trials (17), including trials with NSCLC patients, and its therapeutic benefits have been demonstrated in cancers associated with extremely poor prognoses, such as glioblastomas (40).

ACKNOWLEDGEMENTS

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REFERENCES


23. N. Wauthoz, P. Deleuze, J. Hecq, I. Roland, S. Saussez, I. Adanja, O. Debeir, C.
Decaestecker, V. Mathieu, R. Kiss, K. Amighi. *In vivo* assessment of témozolomide local

24. V. Mathieu, M. Le Mercier, N. De Neve, S. Sauvage, T. Gras, I. Roland, F. Lefranc, R.
Kiss. Galectin-1 knockdown increases sensitivity of temozolomide in a B16F10 mouse

dissolution studies of uranium bearing material in simulated lung fluid. J. Environ. Radioact.

26. V.P. Shah, Y. Tsong, P. Sathe, J-P Liu. *In vitro* dissolution profile comparison-statistics

Belanger, A.A. Brandes, Ch. Marosi, U. Bogdahn, J. Curschmann, R.C. Janzer, S.K. Ludwin,
Radiotherapy plus concomitant and adjuvant temozolomide for glioblastomas. N. Engl. J.

of nanocrystals for solubility and dissolution rate enhancement of nifedipine. Int. J. Pharm.

tobramycin powders containing nanoparticles for pulmonary delivery. Int. J. Pham. 365 : 162-
169 (2009).


32. T. Sebti. Developpement et evaluation de formulations lipidiques à poudre sèche pour inhalation, Thèse (ULB, Pharmacie), Université Libre de Bruxelles, Bruxelles, 2006.


**Table I** Theoretical composition of formulations (F1, F2, F3 and F4) before and after spray-drying

<table>
<thead>
<tr>
<th></th>
<th>Theoretical composition of the suspensions before spray-drying</th>
<th>Theoretical composition of the dry powders after spray-drying</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>F1</strong></td>
<td>TMZ 5%</td>
<td>TMZ 100%</td>
</tr>
<tr>
<td></td>
<td>Isopropanol ad100%</td>
<td></td>
</tr>
<tr>
<td><strong>F2</strong></td>
<td>TMZ 5%</td>
<td>TMZ 95%</td>
</tr>
<tr>
<td></td>
<td>P90H 0.197%</td>
<td>P90H 1.25%</td>
</tr>
<tr>
<td></td>
<td>Cholesterol 0.066%</td>
<td>Cholesterol 3.75%</td>
</tr>
<tr>
<td></td>
<td>Isopropanol ad 100%</td>
<td></td>
</tr>
<tr>
<td><strong>F3</strong></td>
<td>TMZ 5%</td>
<td>TMZ 45.87%</td>
</tr>
<tr>
<td></td>
<td>DLPC 1.4%</td>
<td>DLPC 12.84%</td>
</tr>
<tr>
<td></td>
<td>DMPC 1.4%</td>
<td>DMPC 12.84%</td>
</tr>
<tr>
<td></td>
<td>Lactose 2.5%</td>
<td>Lactose 22.94%</td>
</tr>
<tr>
<td></td>
<td>Phosphate buffer pH 5.0 ad 100%</td>
<td></td>
</tr>
<tr>
<td><strong>F4</strong></td>
<td>TMZ 5%</td>
<td>TMZ 43.29%</td>
</tr>
<tr>
<td></td>
<td>DLPC 1.4%</td>
<td>DLPC 12.12%</td>
</tr>
<tr>
<td></td>
<td>DMPC 1.4%</td>
<td>DMPC 12.12%</td>
</tr>
<tr>
<td></td>
<td>Lactose 3.75%</td>
<td>Lactose 32.47%</td>
</tr>
<tr>
<td></td>
<td>Phosphate buffer pH 5.0 ad 100%</td>
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</table>
Table II The particle size characteristics of raw TMZ, of the TMZ suspensions after HPH processing and of the dry powder formulations (F1, F2, F3 and F4). % < 5 μm (%) and d(0.5) (μm) (mean ± S.D., n = 3) were measured with a Mastersizer 2000® laser diffractometer and 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).

<table>
<thead>
<tr>
<th></th>
<th>Laser light scattering</th>
<th>MsLI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% &lt; 5 μm (%)</td>
<td>d(0.5) (μm)</td>
</tr>
<tr>
<td>Raw TMZ</td>
<td>5.0 ± 0.1</td>
<td>21.3 ± 0.4</td>
</tr>
<tr>
<td>Isopropanolic TMZ suspension after HPH</td>
<td>99.64 ± 0.04</td>
<td>1.50 ± 0.01</td>
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<tr>
<td>Aqueous TMZ suspension after HPH</td>
<td>99.3 ± 0.2</td>
<td>1.527 ± 0.06</td>
</tr>
<tr>
<td>F1</td>
<td>99.17 ± 0.07</td>
<td>1.65 ± 0.01</td>
</tr>
<tr>
<td>F2</td>
<td>97.7 ± 0.4</td>
<td>1.77 ± 0.07</td>
</tr>
<tr>
<td>F3</td>
<td>76 ± 3</td>
<td>1.97 ± 0.09</td>
</tr>
<tr>
<td>F4</td>
<td>72 ± 2</td>
<td>2.75 ± 0.09</td>
</tr>
</tbody>
</table>

Table III Theoretical and actual TMZ content and the moisture content of the dry powder formulations (F1, F2, F3 and F4)
<table>
<thead>
<tr>
<th></th>
<th>Theoretical TMZ content</th>
<th>Actual TMZ content (Mean ± S.D., n=3)</th>
<th>Moisture content (Mean ± S.D., n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>100%</td>
<td>100.5 ± 0.4%</td>
<td>0.3 ± 0.2%</td>
</tr>
<tr>
<td>F2</td>
<td>95%</td>
<td>96 ± 2%</td>
<td>0.24 ± 0.02%</td>
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<tr>
<td>F3</td>
<td>45.87%</td>
<td>77 ± 1%</td>
<td>0.42 ± 0.02%</td>
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<tr>
<td>F4</td>
<td>43.29%</td>
<td>70.6 ± 0.8%</td>
<td>0.58 ± 0.07%</td>
</tr>
</tbody>
</table>
LEGEND TO FIGURES

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

**Fig. 2** DSC heating curves of raw TMZ and of the dry powder formulations, with the temperature noted for the endothermic peak of dehydration of $\alpha$-lactose monohydrate (F3 and F4) and for the exothermic peak of fusion-decomposition of 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, three N°3 HPMC capsules of about 20 mg/test) (mean ± SD, n = 3)

**Fig. 6** Release profiles of TMZ from the dry powder formulations for inhalation (F1, F2, F3 and F4) determined (mean ± S.D., n = 3) after impaction (NGI with an Axahaler® device at 60 l/min, 4 sec, one N°3 HPMC capsule) of 5 mg of particles [dae: 2.82 to 4.46 μm] on the
membrane cassette plunged 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)
Figure 2

Click here to download high resolution image

![Thermal analysis graph showing heat flow versus temperature for different samples labeled F1 to F4 and Raw TMZ.](image-url)
Figure 6

Click here to download high resolution image

Release profiles

![Graph showing cumulative release percentage over time for different formulations](image)

<table>
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<th>30</th>
<th>40</th>
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<tbody>
<tr>
<td>F1</td>
<td>31 ± 4</td>
<td>57 ± 4</td>
<td>78 ± 4</td>
<td>87 ± 2</td>
<td>90 ± 1</td>
<td>92 ± 1</td>
<td>93 ± 1</td>
<td>96 ± 1</td>
<td>96.8 ± 0.5</td>
</tr>
<tr>
<td>F2</td>
<td>35 ± 2</td>
<td>63 ± 4</td>
<td>83 ± 4</td>
<td>90 ± 3</td>
<td>91 ± 2</td>
<td>92 ± 2</td>
<td>92 ± 1</td>
<td>95.6 ± 0.8</td>
<td>96.4 ± 0.3</td>
</tr>
<tr>
<td>F3</td>
<td>36 ± 3</td>
<td>64 ± 4</td>
<td>84 ± 2</td>
<td>89 ± 1</td>
<td>90 ± 1</td>
<td>91.7 ± 0.8</td>
<td>92.6 ± 0.8</td>
<td>95.2 ± 0.3</td>
<td>95.9 ± 0.3</td>
</tr>
<tr>
<td>F4</td>
<td>38 ± 1</td>
<td>65 ± 1</td>
<td>83 ± 4</td>
<td>89 ± 3</td>
<td>91 ± 2</td>
<td>92 ± 2</td>
<td>93 ± 2</td>
<td>94 ± 2</td>
<td>96 ± 2</td>
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