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Assessing the Risk of Formation of Potential Genotoxic Degradation Products in a Small-Molecule Kinase Inhibitor Drug Substance and Drug Product

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ABSTRACT: Galunisertib is a kinase inhibitor designed to selectively inhibit TGF-β signaling. Drug substance stress degradation studies performed during clinical development demonstrated two degradation products via oxidation of the nitrogen(s) of the pyridine moieties in the presence of dilute hydrogen peroxide. These "*N*-oxide" potential degradation products generated positive alerts for mutagenicity by in silico structure−activity relationship-based genotoxicity assessment, and both tested positive in the Ames bacterial mutagenicity test. These compounds were also identified as potential process impurities that could originate through the use of hydrogen peroxide, and this reagent was subsequently removed from the synthetic route. A toxicology limit of not more than 166 ppm (w/w relative to the drug substance) combined for the two *N*-oxides was assigned on the basis of clinical dosing. An LC−MS method was developed to test for the *N*-oxides with a limit of quantitation set at <10% of the toxicology limit in both the drug substance and the drug product tablets. Stability data demonstrated the absence of *N*-oxide formation under long-term and accelerated storage. On the basis of these results, the oxidative degradation pathway was shown to be inactive and nonrelevant for both the drug substance and the drug product.

1. INTRODUCTION

The practices associated with the assessment, testing, and control of impurities with genotoxic potential throughout the clinical phases of development and finalization of drug product (DP) commercial image have evolved through pharmaceutical industry discussions, scientific literature, and the establishment of regulatory guidances. Impurities can be introduced during the drug substance (DS) manufacturing process (process impurities), during storage of the drug substance (DS degradation products), and during drug product manufacturing and storage (DP degradation products). Evaluation of "potential" impurities adds to the complexity of conducting a full genotoxic impurity (GTI) assessment. As outlined in ICH Q3A, a "potential impurity" is one that theoretically can arise during manufacture or storage and may or may not actually appear in the drug substance or the drug product.[1,2] The recently issued ICH M7 guidance[4] provides more clarity on this topic, stating that potential degradation products in the drug substance and drug product are those that may be reasonably expected to form during long-term storage conditions and "include those that form above the ICH Q3A/B identification threshold during accelerated stability studies (e.g., 40°C/75% relative humidity for 6 months) and confirmatory photostability studies as described in ICH Q1B,[3] but are yet to be confirmed in the drug substance or drug product under long-term storage conditions in the primary packaging".[4] Therefore, a holistic GTI control strategy must include an assessment of process capability and control, demonstration of drug substance and drug product stability, and the development of analytical methods for impurity detection and quantification to enable control or discharge of the risk to patient safety. Regulatory guidances specifically focusing on GTIs include a guidance on genotoxic impurities from the European Medicines Agency (EMEA) issued in 2006[5] followed by three rounds of Q&A providing clarification on aspects associated with this guidance,[6] a U.S. Food and Drug Administration (FDA) draft guidance issued in 2008[7] providing direction largely consistent with the concepts outlined in the EMEA guidance, and most recently the International Conference on Harmonization (ICH) M7 Step 4 guidance.[4] Within the industry, a position paper,[8] a review article,[9] several strategy documents,[10−12] and a book[13] have been published on this topic.

Galunisertib is a small-molecule kinase inhibitor designed to selectively inhibit TGF-β signaling, the overexpression of which can enhance tumor growth and exacerbate invasive and metastatic tumor cell behavior.[14,15] The drug product is currently in development in the clinic across a range of cancer and precancer indications. The chemical formula and IUPAC name of galunisertib, respectively, are $C_{22}H_{19}N_5O{\cdot}H_2O$ (MW 387.4) and 4-[2-(6-methylpyridin-2-yl)-5,6-dihydro-4*H*-pyrrolo[1,2-*b*]pyrazol-3-yl]quinoline-6-carboxamide monohydrate (see Figure 1 for the structure). The drug substance

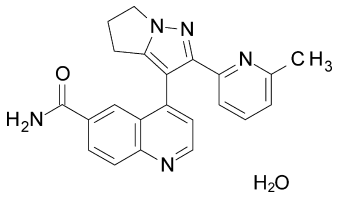

Figure 1. Chemical structure of galunisertib.

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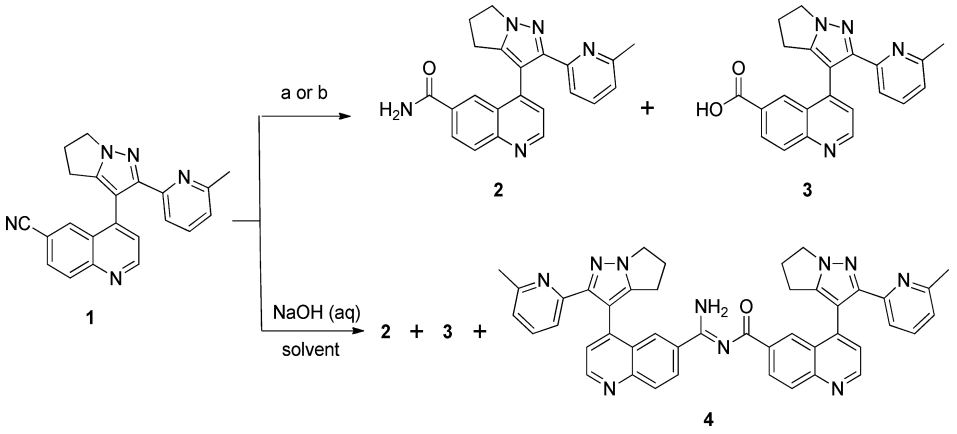
Scheme 1. Reaction products observed in the hydrolysis of 1 to 2[17,a]



[a]Reaction conditions: (a) K_2CO_3, 35% H_2O_2, H_2O, DMSO, carbon treatment (87% yield in campaign 1); (b) NaOH, 35% H_2O_2, H_2O, NMP, DMSO (87% yield in campaign 2).

exists as a crystalline monohydrate that is stable across a wide range of temperature and humidity conditions bracketing an ambient environment. The drug substance possesses two pK_a values corresponding to the conjugate acids of the weakly basic moieties in the structure (pK_{a1} = 3.30, pK_{a2} = 4.43) and is practically insoluble in water or aqueous solutions at neutral and alkaline pH and slightly soluble at low pH and in methanol, ethanol, and isopropyl alcohol. The drug substance manufacturing process uses three starting materials, consists of three synthetic steps, and employs three covalent-bond-forming/breaking reactions. The process produces a high-purity crystalline drug substance.

Recently the ICH M7 Guideline "Assessment and Control of DNA Reactive (Mutagenic) Impurities in Pharmaceuticals To Limit Potential Carcinogenic Risk" has been established.[4] The purpose of this guideline is to provide a practical framework that is applicable to the identification, categorization, qualification, and control of GTIs to limit potential carcinogenic risk. Because of the broad range of clinical studies associated with galunisertib, the compound does not fall within the narrower scope of the ICH S9 guidelines for drug substances and drug products intended exclusively for advanced cancer with limited therapeutic options.[16] This report describes stress-testing studies to help determine potential degradation products of galunisertib. The stress evaluation demonstrated the formation of *N*-oxides from exposure to peroxides, a degradation pathway that demonstrated overlap with potential side products from the manufacturing process (peroxide oxidative hydrolysis of the nitrile precursor).[17] In alignment with the M7 guidance, the in silico assessment of the *N*-oxides for genotoxic potential was followed by isolation/purification, Ames testing, and the establishment of a control strategy.

2. RESULTS AND DISCUSSION

A. Drug Substance Manufacturing, Stress Degradation, and Stability. *Drug Substance Manufacturing Process Development.* The development of a robust, highly selective, aqueous, base-catalyzed process for the penultimate step in the preparation of galunisertib (**2**) has been described elsewhere.[17] Specifically, early process development efforts indicated that hydrogen peroxide-mediated hydrolysis of a nitrile-containing precursor to form the amide moiety would be superior to a simple acid- or base-catalyzed process because of better control of undesired "overhydrolysis" to give the acid impurity **3** (Scheme 1, conditions a). This process was further refined for scale-up by using a mixed *N*-methyl-2-pyrrolidone (NMP)/dimethyl sulfoxide (DMSO) solvent system to reduce sulfur emissions and substituting sodium hydroxide for potassium carbonate to enhance base solubility (Scheme 1, conditions b). While this process was relatively robust, safety concerns involving hydrogen peroxide, oxygen generation during the process, and increased concern regarding incineration of sulfur-containing waste streams on a commercial scale prompted the pursuit of an alternative peroxide-free process. To address these issues, a simple aqueous base reaction system was developed to carry out the conversion of **1** to **2** utilizing NMP as a sacrificial solvent to minimize product loss due to hydrolysis and eliminate the potential formation of genotoxic *N*-oxides.[17]

Drug Substance Stress Degradation and Structural Characterization. Because ICH M7 requires an assessment of potential impurities (i.e., those "reasonably likely to occur")[5,7] and shelf-life-aged samples were not available, the potential degradation products identified during stress-testing studies (i.e., forced degradation studies) were evaluated for mutagenic potential.[12,18,19] Therefore, in parallel to the development of an optimized chemical synthesis process, the stability of galunisertib drug substance in the solid state and in aqueous solutions was assessed using stress-testing studies according to ICH and EMEA guidelines[1,20,21] and published experimental strategies;[22,23] such studies are useful for enabling the development of valid stability-indicating analytical methods. Samples of the drug substance in the solid state were stressed under various conditions of heat, relative humidity (RH), and light, as were samples in aqueous solution across the pH range from 1 to 13 using acetonitrile as a cosolvent to facilitate solubility. The drug substance was found to be stable in the solid state after exposure to 70 °C/75% RH for 21 days and simulated sunlight for a total exposure of approximately 1800 W h m^{-2} (UVA) and 3 × 10^6 lux h (visible). In solution, degradation was found to occur through conversion of the amide moiety to its corresponding carboxylic acid, especially at low pH, and also through exposure to light at both low and high pH. The strategy chosen by Eli Lilly has been to perform structural elucidation of the major potential degradation products from stress-testing studies[22,23] and then submit these structures for genotoxic risk assessment.[12]

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The susceptibility of galunisertib to oxidative degradation was evaluated under the following three stress conditions through storage at 27 °C for time periods ranging from 8 h up to 5 days: (1) transition-metal-catalyzed oxidative degradation using solutions of the drug substance in the presence of Fe(III) or Cu(II) sulfate salts, (2) free-radical-induced oxidative degradation using the radical initiator 2,2′-azobis(2,4-dimethylpentanenitrile) (VAZO 52) in solution, and (3) peroxide-mediated oxidative degradation in the presence of 0.3% hydrogen peroxide in solution. No significant degradation was observed in samples exposed to the iron or copper salts, indicating that galunisertib is not susceptible to metal-catalyzed degradation. The compound was also found to experience only minor free-radical-induced oxidative degradation. However, significant degradation of galunisertib was observed in aqueous/acetonitrile solutions containing dilute hydrogen peroxide. Two major degradation products were generated under these conditions, and following preparative isolation and spectroscopic characterization, these degradants were identified as the quinoline *N*-oxide and pyridinium *N*-oxide forms of galunisertib. For the purpose of this report, these two compounds will be called "*N*-Oxide 1" and "*N*-Oxide 2", respectively (see Figure 2).

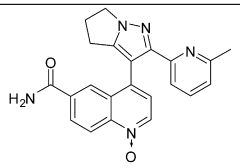
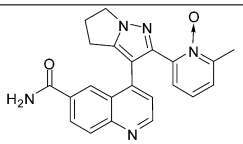
Impurity Name	Structure
Quinoline N-oxide (N-Oxide 1)	 Chemical Formula: $C_{22}H_{19}N_5O_2$ Exact Mass: 385.1539
Pyridinium N-oxide (N-Oxide 2)	 Chemical Formula: $C_{22}H_{19}N_5O_2$ Exact Mass: 385.1539

Figure 2. Chemical structures of galunisertib degradation product compounds *N*-Oxide 1 and *N*-Oxide 2.

Evaluation of Toxicological Risk and Establishment of Control Strategy. In accordance with FDA and ICH M7 Guidances,[4,7] a computational toxicological assessment can be used to reveal quantitative structure−activity relationships (QSARs) as the first step in an evaluation of the genotoxicity and carcinogenicity of compounds. Two QSAR prediction methodologies that complement each other should be applied: one methodology should be expert-rule-based, and the other should be statistics-based. Therefore, together with the chemical structures of the other potential degradants of galunisertib, the structures of *N*-Oxide 1 and *N*-Oxide 2 were subjected to in silico analyses to screen for toxicologically relevant "alerts" using the databases Deductive Estimation of Risk from Existing Knowledge (DEREK), version 13 (Lhasa, Leeds, UK) and Leadscope Genotox Experts Alerts Suite (LS Model Applier Version 1.8.3, Salmonella and *E. coli* Mutagenicity Models, Leadscope, Inc., Columbus, OH). The DEREK analysis indicated that among the set of degradation product structures, only *N*-Oxide 1 resulted in a positive alert for mutagenicity. Conversely, only the *N*-Oxide 2 structure resulted in a positive alert for mutagenicity by the Leadscope evaluation. These assessments were followed up with preparative isolation of both *N*-Oxide 1 and *N*-Oxide 2 and their evaluation in the Ames test, a biological assay considered to be indicative of mutagenicity.[24] Positive results for both compounds in the Ames test indicated both aromatic *N*-oxide compounds to be mutagenic as "class 2" impurities according to the ICH M7 classification (known mutagens with unknown carcinogenic potential).[4] The Ames results were not surprising considering that aromatic *N*-oxides are one of the Ashby−Tennant structural alerts corresponding to mutagenicity.[25] Therefore, the development of a manufacturing process and stability control strategy was warranted, since as described earlier these compounds were also identified as potential synthetic-route process impurities.[17] A toxicology limit of not more than 166 ppm (w/w relative to galunisertib) combined for *N*-Oxides 1 and 2 was assigned on the basis of a clinical dose of 300 mg of galunisertib daily for 2 years and a modified threshold of toxicological concern (TTC) approach appropriate for oncolytic agents justifying a maximum tolerable dose of 50 μg/day.[11] This limit was established in lieu of the commonly applicable TTC of 1.5 μg/day.[5] The limit was initially set prior to the implementation of the ICH M7 guidance, which discounts synergistic effects between multiple mutagens. Therefore, the 166 ppm toxicology limit established for this study may be considered overly conservative, but it was left in place long-term because the method and controls were demonstrated to effectively support this limit.

According to the EMEA, if a GTI is introduced or formed in the last synthetic step, it should be included as a drug substance specification unless chemical testing data demonstrate that the impurity consistently does not exceed 30% of the limit.[6] In the absence of regulatory guidance on the control of GTIs that are only potential degradation products, a more conservative 10% limit was adopted to support full discharge of the risk associated with the presence of the *N*-oxide compounds. Initial attempts to develop a UV−HPLC method to measure *N*-Oxides 1 and 2 at these levels were unsuccessful because although adequate sensitivity was obtained using this approach, acceptable specificity was not achievable. It was presumed, however, that on the basis of this evaluation UV-HPLC most likely could serve adequately as a qualitative "pass/fail" limit test based on the 166 ppm toxicology limit if this approach was desired at some point in the future. To meet the current objective, an LC−MS method was subsequently developed to quantitate the *N*-oxides to levels <10% of the combined limit (i.e., <16 ppm) in both the drug substance and the drug product tablets (see the Experimental Section).

Stability Assessment for N-Oxide GTI Formation. As indicated in the ICH M7 guidance, for potential degradation products that have been characterized as mutagenic, it is important to understand whether the degradation pathway is relevant to the drug substance and drug product manufacturing processes and their proposed packaging and storage conditions.[4] Therefore, galunisertib drug substance and drug product samples were evaluated for levels of *N*-Oxides 1 and 2 under long-term and accelerated storage conditions according to ICH Q1A stability study guidelines.

Two drug substance batches were placed on stability. Batch 1 was manufactured using the synthetic route that utilized hydrogen peroxide for the step 2 hydrolysis, while batch 2 was manufactured using the aqueous base reaction system as a replacement for hydrogen peroxide. Testing of drug substance

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batch 1 after storage in the container closure system used for clinical material (4 mil Armorflex 104 LLDPE liner (ILC Dover, Frederica, DE) in a secondary laminated aluminum foil liner) and in the container closure system intended for commercial use (4 mil Armorflex 114 LLDPE liner (ILC Dover) in a secondary laminated aluminum foil liner) for 6 months on accelerated storage (40 °C/75% RH) and 36 months on long-term storage (25 °C/60% RH) revealed a trace level of *N*-Oxide 1 (8−11 ppm) that did not change on stability, while levels of *N*-Oxide 2 remained at less than 8 ppm at the initial time point and throughout the length of the study. Batch 2 drug substance samples were packaged in similar containers and demonstrated levels of both *N*-Oxides 1 and 2 to be less than 8 ppm on stability at the initial time point and throughout 6 months on accelerated storage and 24 months on long-term storage. Therefore, the drug substance stability results demonstrated that *N*-Oxides 1 and 2 not only were eliminated as process impurities through the removal of hydrogen peroxide as a reagent but also did not form over time during storage in the solid state.

B. Drug Product Manufacturing and Stability. *High-Shear Wet Granulation Formulation.* Following the confirmation of *N*-Oxides 1 and 2 as potential genotoxic impurities, high-shear wet granulation (HSWG) formulation drug product clinical trial batches manufactured using drug substance batch 1 (see section 4B for a qualitative formula) were removed from stability chambers and tested. These materials represented 10, 50, and 100 mg dose strengths and had been stored for approximately 68 months at 25 °C/60% RH. The results from the testing indicated *N*-Oxide 1 to be present at levels less than 8 ppm across the three dose strengths, while the levels of *N*-Oxide 2 were determined to be 13 ppm, <8 ppm, and <8 ppm in the 10, 50, and 100 mg tablets, respectively. These results demonstrated that the *N*-oxides did not form to any appreciable extent as a result of either the manufacturing process or during storage.

Roller Compaction Formulation. Commercial development efforts resulted in a second formulation using a roller compaction (RC) platform in 80 mg and 150 mg tablets. As was the case for the HSWG formulation, the RC formulation (see section 4B for a qualitative formula) utilized excipients that did not contain peroxide impurities, and unlike the HSWG formulation, the RC process is a dry process, thus even further reducing the risk of *N*-oxide formation. Tablets representative of the proposed commercial formulation were exposed to stress conditions. Samples stored at 70 °C/75% RH for 30 days and samples exposed to simulated sunlight (~168 000 lux visible intensity and ~64 W/m^2 near-UV) for 16 h were stable and did not exhibit an increase in impurity levels, including compounds **2** and **3**. Batches of 80 mg and 150 mg RC tablets produced with drug substance batch 2 and representative of the proposed commercial formulation were monitored for *N*-Oxides 1 and 2 through exposure to the same stress conditions just described and also on long-term stability tests. The stability data demonstrated no measurable levels of *N*-oxides (i.e., <8 ppm) after exposure to the stress conditions and also at the initial time point, through 6 months on accelerated storage (40 °C/75% RH), and through 12 months on long-term storage (30 °C/65% RH). These results confirmed that the potential degradation products *N*-Oxide 1 and *N*-Oxide 2 do not have an active degradation pathway in the commercial tablet formulation and will not form at levels approaching the acceptable limit under the proposed packaging and storage conditions.

According to ICH M7, a "well-designed accelerated stability study (e.g., 40 °C/75% RH, 6 months) in the proposed packaging" can be used to determine the relevance of a potential degradation product that is genotoxic.[4] Furthermore, ICH M7 states that "based on the result of these accelerated studies, if it is anticipated that the degradation product will form at levels approaching the acceptable limit under the proposed packaging and storage conditions, then efforts to control formation of the degradation product are expected. In these cases, monitoring for the drug substance or drug product degradation product in long term primary stability studies at the proposed storage conditions (in the proposed commercial pack) is expected unless otherwise justified." The guidance seems to clearly indicate that additional monitoring of this pathway in long-term primary stability studies is not needed if the results from the accelerated and long-term studies indicate that the degradation pathway is inactive, as we have shown in this case.

3. CONCLUSIONS

To date, long-term stability studies through 36 months and 24 months, respectively, for the drug substance batches manufactured using both the initial synthetic route and the proposed commercial route (removing hydrogen peroxide from the penultimate step) have demonstrated no increase in the levels of the two *N*-oxide impurities, with no detectable amounts (<8 ppm) of the two *N*-oxide impurities in the latter process. Similar stability in regard to the absence of *N*-oxide impurity level formation in the drug product has been indicated by long-term stability data for both the clinical trial HSWG formulation and the proposed commercial RC formulation through 68 months and 12 months, respectively, and also through 6 months under accelerated storage conditions for the two formulations and short-term solid-state stress conditions for the latter. On the basis of the results of these studies, the evidence demonstrated that neither of the *N*-oxides forms on stability. In addition to the removal of hydrogen peroxide from the synthetic route, which was demonstrated to prevent formation of the *N*-oxides during the drug substance manufacturing process, the oxidative degradation pathway was shown to be inactive and nonrelevant for both the drug substance and the drug product in the solid state.

4. EXPERIMENTAL SECTION

A. Drug Substance Stress Degradation and Degradant Characterization. Solution samples were prepared by dispensing approximately 4.5 mg of galunisertib drug substance into a 20 mL scintillation vial, accurately weighing the sample, and adding the appropriate solvents, including acetonitrile, to an approximate final concentration of 50%. The hydrogen peroxide-containing samples were prepared by adding 1.5 mL of 3% hydrogen peroxide and diluting to a final volume of 15 mL with water and acetonitrile, resulting in a final hydrogen peroxide concentration of approximately 0.3%. Samples containing VAZO 52 were prepared by diluting 1 mL of a 15 mg/mL solution of VAZO 52 in water and acetonitrile to a final volume of 15 mL, resulting in a final VAZO 52 concentration of approximately 1 mg/mL. The Fe(III)- and Cu(II)-containing samples were prepared by diluting 1 mL of a 15 mM solution of ferric or cupric sulfate to a final volume of 15 mL with water

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and acetonitrile, resulting in a final Fe(III) or Cu(II) concentration of approximately 1 mM. The final volume of all stock solutions was 15 mL. The prepared stock solutions were dispensed into 2 mL HPLC vials for storage. Samples were removed from their respective stress conditions at specified intervals and stored at 4 °C until all of the samples had been pulled. All of the solution dispenses and dilutions were tracked gravimetrically. In addition, any solvent evaporation from the stressed solutions during storage was taken into account by accurately weighing the individual samples before and after stressing.

The major degradation products[22,23] observed in this study were characterized using an Acquity UPLC instrument (Waters Corporation, Milford, MA) and an Acquity BEH C18 column (2.1 mm × 100 mm, 1.7 μm) at 45 °C with an "A" mobile phase of 25 mM potassium phosphate (pH 6.5) and 5% acetonitrile and a "B" mobile phase of acetonitrile, eluting with a 6 min gradient from 2 to 70% B at 0.6 mL/min. The sample concentration and injection volume for this analysis were 0.3 mg/mL and 0.8 μL, respectively, and the separation profile was monitored at 225 nm using an Acquity UPLC photodiode array detector (Waters). Select samples exhibiting significant degradation peaks were analyzed using UPLC−MS. The UPLC instrument was coupled to a Waters SQD mass spectrometer equipped with an electrospray ionization (ESI) source. The instrument was programmed to switch between positive and negative ion mode during data collection to yield both positive and negative ion electrospray results. Mass spectra were correlated to degradation peaks by retention time and UV spectral match. Baseline correction was utilized to simplify the spectra. The UPLC−MS conditions were identical to those described above with the exception of the "A" mobile phase, which consisted of 25 mM ammonium acetate (pH 5.6) and 5% acetonitrile. Mass spectra were acquired at 1 scan/s over a scan range of m/z 100−1000. A source temperature of 150 °C was used, along with positive/negative ion capillary voltages of 0.60/0.96 kV and cone voltages of 40/48 kV. Nitrogen was employed as both the sheath gas and auxiliary gas. Data collection and processing were performed on a PC using Empower 3 chromatography data software.

The two oxidative degradation products were generated by preparing solutions of galunisertib in the presence of peracetic acid. Approximately 290 mg of drug substance was dissolved in 100 mL of 50/50 H_2O/acetonitrile containing 1.0 mL of peracetic acid, and the solution was held under ambient conditions for 1 day. Confirmation of the identities of the degradants was achieved by UV photodiode array and mass spectral comparison to library spectra generated from the original stress test samples. Isolation of the two components was carried out using preparative reversed-phase chromatography. Fractions were collected, pooled, and then frozen and lyophilized to remove solvent. Approximately 5 mg of each degradant was obtained and submitted for spectroscopic characterization via direct-infusion high-resolution mass spectrometry and NMR spectroscopy. The high-resolution mass spectra indicated a gain of one oxygen to galunisertib in each structure, and ^{1}H NMR analysis provided the structures displayed in Figure 2.

B. Drug Product Tablet Formulation. The initial galunisertib formulation for clinical trial supply consisted of an HSWG process involving tray drying and tablet compression with no film coating to provide 10, 50, and 100 mg strength tablets. In this formulation, the drug substance, lactose monohydrate, microcrystalline cellulose, starch, and a portion of croscarmellose sodium were dry-blended in a granulator, and then a granulation solution consisting of hydroxypropyl methylcellulose in purified water was sprayed onto the powders while mixing to a suitable granulation end point. The granulation was wet-sieved and dried, and the size of the granulation was reduced by passage through a comill. These powders were blended with croscarmellose sodium and magnesium stearate and then compressed into tablets. The tablets were not film-coated and were packaged in PCTFE (Aclar) blisters for clinical use. For the assessment of drug product stability, the accelerated storage conditions were 40 °C/75% RH and the long-term storage conditions were 30 °C/65% RH.

To support potential commercialization and to enhance convenience for patients and caregivers, a roller compaction (RC) manufacturing process and unit composition was developed as a second formulation. The RC manufacturing process consisted of standard tableting excipients: mannitol spray-dried, microcrystalline cellulose, croscarmellose sodium, colloidal silicon dioxide, and magnesium stearate. The magnesium stearate concentration was adjusted across the range of 1.3% to 2.4% through several batch manufactures in an effort to optimize the lubrication during the process. These materials were blended, roller-compacted, and then mixed with extragranular powders and compressed at two different weights to produce core tablets of the desired drug content (80 mg and 150 mg). The core tablets were film-coated with two different shades of a soluble film coating to differentiate further the two dosage strengths. The resulting tablets were packaged in Aclar blisters or plastic bottles for use in the clinical program. For the assessment of drug product stability, the accelerated storage conditions were 40 °C/75% RH and the long-term storage conditions were 30 °C/65% RH.

C. Analytical Method Procedure and Validation. Drug substance samples were prepared in a 10 mL flask at a nominal concentration of 2 mg/mL through solubilization of approximately 20 mg of solids in 0.1% formic acid in 50% methanol (sample solvent). Drug product samples were prepared at the same nominal concentration in sample solvent by dissolution of an appropriate number of tablets in a volumetric flask through the use of a wrist-action shaker for 60 min, followed by filtration through a 0.45 μm pore size, 25 mm diameter PTFE acrodisc CR syringe filter (Pall Corporation, Port Washington, NY). Stock solutions of *N*-Oxide 1 and 2 standards were prepared in sample solvent at a concentration of 0.016 mg/mL. The stock solution was diluted further to make a working standard solutions equivalent to 0.320, 0.160, and 0.016 μg/mL, corresponding to levels of 160, 80, and 8 ppm w/w based on the nominal concentration of galunisertib. The low standard of 8 ppm was intentionally established to represent 1/10 of the TTC limit for the two combined *N*-oxide impurities assuming equal concentrations in the absence of knowledge regarding preferential formation of either impurity. The samples and standards (5 μL injections) were analyzed by HPLC−ESI-MS on an Agilent (Santa Clara, CA) 1100 HPLC system interfaced with a model 6130 quadrupole mass spectrometer operated by ChemStation software. Samples were stored in a refrigerated 5 °C autosampler prior to injection. Separations were performed using a reversed-phase XBridge (Waters, Milford, MA) Phenyl column (2.5 μm, 4.6 mm × 75 mm) together with an "A" mobile phase consisting of 0.1% formic acid in water and a "B" mobile phase consisting of 0.1% formic acid in methanol. The

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initial mobile phase composition was 25% B with a gradient to 30% B over the course of 5.0 min. A 4.0 min hold at 75% B at the end of the gradient was followed by a 6.0 min re-equilibration at 25% B for a total run time of 15.0 min. The flow rate remained constant at 1.0 mL/min throughout the run. ESI mass spectral data were collected using positive ion selected ion monitoring (SIM) mode at m/z 386 at the following settings: fragmentor voltage, 70 V; gain EMV, 1.0; dwell time, 590 ms; gas temperature, 350 °C; vaporizer temperature, 200 °C; drying gas flow rate, 12.0 L/min; nebulizer pressure, 50 psi; capillary voltage, 3000 V. Peak areas were integrated using the ChemStation software, and least-squares regression was employed to generate a linear calibration curve for peak area versus standard concentration. No blanks were used for curve fitting, and the line was not forced to go through zero. From the analysis, any result less than the low standard of 8 ppm (1/10 of approximately half the TTC limit for the two combined *N*-oxide impurities) was reported as "less than 8 ppm".

A limited method validation appropriate at this clinical stage of project development was conducted for testing of the drug substance and the RC drug product according to the ICH guidelines.[26] Specificity was evaluated by injecting the 8 ppm standards, sample solvent, and drug product placebo, and no significant interference from components of the sample solvent or the tablet excipients was observed (see Figure 3). Linearity

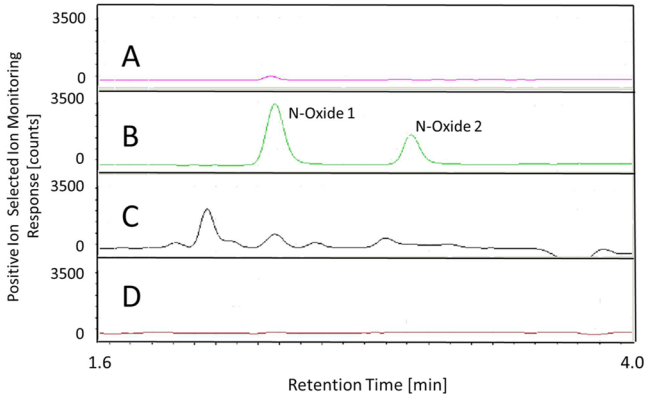

Figure 3. Total ion ESI-MS chromatograms of (A) a sample solvent blank, (B) the 8 ppm *N*-Oxides 1 and 2 mixed standard, (C) a commercial RC formulation drug product tablet, and (D) placebo.

was assessed using solutions at five concentrations across the range of 8−160 ppm and provided a correlation coefficient (r) of 0.999 for both target compounds. For the drug substance matrix, accuracy was determined through analyses of both analytes spiked in triplicate into drug substance at levels of approximately 8 and 150 ppm with recoveries ranging from 89% to 112% and precisions of 1.4% and 1.3%, respectively, for *N*-Oxide 1 and 2.0% and 1.6% respectively for *N*-Oxide 2. For the drug product matrix, accuracy was determined through analyses of both analytes spiked in triplicate into placebo at levels of 8, 80, and 160 ppm with recoveries ranging from 100% to 125% and precisions of 15.0%, 1.4%, and 1.3%, respectively, for *N*-Oxide 1 and 4.5%, 1.2%, and 1.6%, respectively, for *N*-Oxide 2. Filtered sample recoveries were determined to be within 10% of samples clarified by centrifugation as controls. In the drug substance matrix, the detection and quantitation limits were determined (via signal-to-noise evaluation of the 8 ppm standard in comparison with a blank) to be 0.07 and 0.2 ppm, respectively, for *N*-Oxide 1 and 0.2 and 0.6 ppm, respectively, for *N*-Oxide 2. In the drug product matrix, the detection and quantitation limits were determined to be 0.4 and 1.3 ppm, respectively, for *N*-Oxide 1 and 0.8 and 2.6 ppm, respectively, for *N*-Oxide 2. The standard and sample solutions were determined to be stable (recoveries within the range of 80−120% of initial) for at least 24 h when stored under ambient conditions and for at least 72 h when stored at 5 °C.

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Notes

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