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Characterization of degradation products of regorafenib by LC-QTOF-MS and NMR: Investigation of rearrangement and odd-electron ion formation during collision-induced dissociations under ESI-MS/MS

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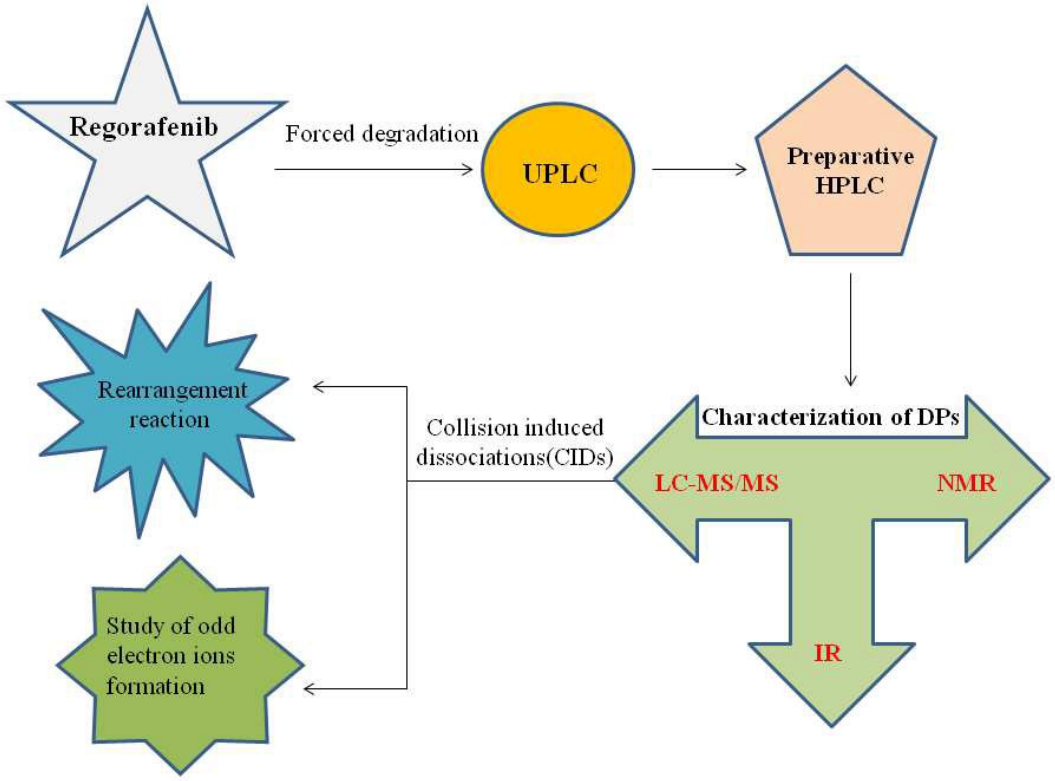
Graphical abstract



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Abstract

Regorafenib is an oral multikinase inhibitor, was subjected to stress conditions (hydrolysis, oxidative, thermal and photolytic) as per ICH specified conditions. The drug showed considerable degradation under hydrolysis (acidic, basic and neutral) and oxidative stress conditions, whereas it was stable under other stress conditions. A total of five degradation products (DPs) were observed and these were analyzed by using UHPLC-DAD system. The chromatographic separation was achieved on an Acquity CSH C18 (100×2.1 mm, 1.7µ) column using 0.1% formic acid and acetonitrile: methanol (80: 20, %v/v) as mobile phase in gradient mode. All DPs were characterized by LC–MS/MS and major degradation product (DP1) was isolated by using preparative HPLC from degradation mixture and analyzed using NMR (1D and 2D NMR) and IR experiments. It was observed that protonated DP1 and DP3 undergo rearrangement reactions during collision induced dissociations under positive electrospray ionization conditions. Additionally, *in silico* toxicity of the drug and its degradation products (DP1-DP5) were evaluated using TOPKAT and DEREK toxicity prediction software tools.

Keywords: Regorafenib; Stress study; Characterization; Rearrangement reaction; Odd-electron ion

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Introduction

According to the American Cancer Society, cancer is the second most common cause of death and it accounts for nearly one out of every 4 deaths. Cancer is defined as a disease caused by an uncontrolled division of abnormal cells in a part of the body. In this condition, the cells of a specific tissue lose their ability to respond to the signals within the tissue that regulate cellular differentiation, survival, proliferation, and death. As a result, these cells accumulate causing local damage and inflammation. There are over 200 different types of cancer, and each is classified by the type of cell that is initially affected.[1]

Of all the medications that have come out to treat this condition, kinase inhibitors have played an increasingly prominent role. Phosphorylation regulates many biological processes of our body, and protein kinase inhibitors can be used to treat diseases due to hyperactive protein kinases (including mutant or over expressed kinases in cancer) or to modulate cell functions to overcome other disease drivers.[2]

Regorafenib [4-[4-({[4-Chloro-3-(trifluoromethyl)phenyl]carbamoyl}amino)-3-fluorophenoxy]-N-methylpyridine-2-carboxamide hydrate] is a novel oral multikinase inhibitor that potently inhibits the endothelial cell kinases in biochemical and cellular kinase phosphorylation assays. It also inhibits additional angiogenic and the mutant oncogenic kinases (KIT, RET, and B-RAF).[3-5]

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Forced degradation or stress degradation study include degradation of a new drug substance and drug product under different stress conditions more harsher than accelerated conditions and it provides an insight into determination of degradation pathways, degradation products (DPs), intrinsic stability of a drug substance, structure elucidation of DPs and also essential for demonstrating specificity of stability indicating methods. These forced degradation studies are also helpful for a demonstration of the chemical behavior of the drug molecule which in turn used in the formulation development and final package. [6-8]

The FDA and other regulatory agencies state the stability testing data as a regulatory requirement and it has been extended to generic drugs. [9,10] The degradation products formed under forced degradation studies may or may not mimic process impurities [11-14] and real time storage impurities. However, the developed stability indicating method can also be applied for the analysis of samples generated under long term, accelerated stability studies and real time storage conditions. [6,9]

Recently there have been reports on synthesis of REG,[15,16] HPLC method,[17] and LC-MS assay method for determination of regorafenib in rat plasma.[18-20] However, no work has been published on forced degradation studies of regorafenib and characterization of its degradation products. Hence, the purpose of this study is to identify and characterize the degradation products of regorafenib using LC/ESI/QTOF/MS/MS, NMR and IR experiments and to evaluate *in vitro* toxicities of degradation products.

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Experimental

Chemicals and reagents

Pure regorafenib was obtained as gratis sample from MSN Laboratories (Hyderabad, India). High-performance liquid chromatography (HPLC) grade acetonitrile (ACN) and methanol (MeOH) were purchased from Merck (Merck, Mumbai, India). Buffer salts and all other chemicals such as formic acid, ammonium acetate, hydrochloric acid, sodium hydroxide and 30% (w/w) hydrogen peroxide were purchased from SD Fine Chemicals Pvt. Ltd., (Mumbai, India). Ultra pure water (H_2O) was obtained from a Millipore Milli-Q plus system (Milford, MA, USA)

Instrumentation

UPLC (Ultra Performance Liquid Chromatography) studies were carried out on Waters UPLC® H-class system (Waters Corp., Milford, MA, USA), which was equipped with an auto sampler and quaternary gradient pump with an in-line degasser, multiple column compartments and photodiode array (PDA) detector. The chromatographic data were acquired using Empower 3 software (Waters Corp.).

LC-HRMS (Liquid Chromatography- High Resolution Mass Spectrometry) studies were carried out using an Agilent Infinity 1290 series instrument (Agilent Technologies, Santa Clara, California, USA) comprising of

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quaternary pump (G4204A), auto sampler (G4226A), column oven (G1316C), thermostat (G1330B) and PDA detector (G4212B). The HRMS system consisted of quadrupole time-of-flight (Q-TOF LC–MS 6540) equipped with an electrospray ionization (ESI) source. System control and data acquisition were done by Mass Hunter Workstation software.

Isolation of selected DP was carried out by using semi preparative HPLC instrument (Waters Corp.), which is equipped with a binary pump (515 and 515), a de-gasser, pump control module, manual injector, a diode-array detector (2489 UV-VIS), and fraction collector. The data acquisition was done under the control of Empower 3 software (Waters Corp.).

NMR (Nuclear magnetic resonance) experiments (1D and 2D NMR) were done by AVANCE III HD-500 MHz NMR, Bruker (Bruker, Billerica, Massachusetts, USA). DMSO-d_6 was used as solvent. ^{1}H and ^{13}C chemical shift values were reported on the δ scale in ppm relative to TMS (δ = 0.00 ppm) as an internal standard. The data acquisition and processing of NMR spectra was done using Top spin software (3.2 version). The FTIR (Fourier transform infrared spectroscopy) spectra were recorded by using PerkinElmer Spectrum (Two spectrophotometer, PerkinElmer, Inc., Waltham, MA, USA). Photolytic studies were performed in a photostability chamber (Osworld Scientific Pvt. Ltd., Mumbai, India) and thermal degradation studies were carried out in the Osworld laboratory oven (Osworld Scientific Pvt. Ltd.). Eutech pH meter (Eutech Instruments, Singapore) was used to evaluate the pH of all the solutions. To

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dissolve the samples, Power Sonic-405, an ultra-sonicator (Hwashin Technology Co., Seoul, South Korea) was used.

Sample preparation

The diluent (ACN: water (80: 20, % v/v)) was used to prepare the standard as well as stress degradation samples. The acidic and basic stressed degradation samples were neutralized and diluted to 100ppm with the diluent before UPLC analysis.

Forced degradation studies on REG

Forced degradation of REG was performed on the bulk drug (API) as per ICH guidelines.[21] REG was subjected to different stress conditions such as hydrolysis (acid, base and neutral), oxidative, photolytic and thermal stress conditions. The drug solutions were prepared at 2.0 mg/mL concentration for all stress samples and diluted with acid, base and 30% H_2O_2 in 1: 1 ratio. All the optimized stress conditions are given in Table 1.

Table 1 Stress conditions for optimum degradation of REG

Stress condition	Concentration of stressor	Exposed condition	Duration	Degradation products formed
Acid hydrolysis	2 N HCl	80 °C	5 h	DP1, DP2 and DP3
Base hydrolysis	0.1 N NaOH	80 °C	24 h	DP1, DP2 and DP4
Neutral hydrolysis	Diluent	80 °C	3 d	DP1 and DP2
Oxidation	30% H_2O_2	RT	6 d	DP3 and DP5
Thermal	Solid state	100 °C	15 d	No degradation
Photolysis	Solid state	40 ◦C, 75% RH	4 d	No degradation

Enrichment, isolation and NMR studies on major degradation product DP1

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The major degradation product DP1 was observed in all the hydrolytic (acid, alkaline and neutral) stress conditions. Therefore neutral hydrolytic condition was tried to enrich major degradation product DP1 by dissolving 150 mg of REG in 50 mL of diluent and refluxed for 120 h at 75 °C, where DP1 was formed in major quantity. The degradation product (DP1) was separated on a preparative column (X-bridge C18 (250 mm × 19 mm, 5 µm)), by using a mobile phase composed of 0.1% formic acid (A) and acetonitrile (B) in an isocratic mode (35: 65, % v/v). The optimized flow rate, injection volume, and detection wavelength were 12 mL/min, 1.5 mL and 261 nm, respectively. The fraction (DP1) was collected and concentrated by using rotavapor. The isolated products were dissolved in deuterated DMSO and subjected to ^{1}H and ^{13}C NMR experiments.

Fourier transform infrared spectroscopy (FTIR)

The FTIR spectra of REG and DP1were recorded in the range of 4000–400 cm^{-1}. A small quantity of dried powder sample of REG and DP1 were placed on ATR crystal and recorded FTIR spectra for functional group determination.

***In silico* toxicity prediction**

In silico models offer an economical, fast and easy way of evaluating toxicological properties of a drug molecule prior to synthesis. Several commercial toxicity prediction tools available for toxicity prediction, covering a range of toxicity endpoints such as carcinogenicity, reproductive effects, irritation, hepatotoxicity, teratogenicity, and mutagenicity etc. Two types of

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approaches are available to predict the toxicity of molecules such as knowledge-based and statistically based systems. A knowledge-based system (DEREK, Deductive Estimation of Risk from Existing Knowledge) uses human expert opinion to predict the potential toxicity of new molecules based on relationship between structure and biological activity, whereas statistically based systems (TOPKAT, Toxicity Prediction by Komputer Assisted Technology) use application of various statistical methods, calculated parameters, and structural connectivity to derive the toxicity data for molecules.[22,23] In the present study two computational tools (TOPKAT (Discovery Studio 2.5, Accelrys, Inc., San Diego, CA, USA) and DEREK (Nexus v2.0, Lhasa Ltd., Leeds, UK)) were used to evaluate the toxicity of REG and its degradation products.

Results and discussion

Optimization of chromatographic conditions

Initial trials were done with ammonium acetate buffer at different pH (3.5, 4.0, 5.0 and 6.5 pH) conditions on BEH C18 column for optimum separation of REG and its DPs. It was observed that peak shapes were not good. Then moved to 0.1% formic acid from ammonium acetate which gave better peak shapes. At this stage DP3 co-eluted with DP5. To get better separation between the DP3 and DP5, methanol was added to organic phase B (acetonitrile). Finally REG and its DPs were separated on an Acquity CSH C18 (100 mm × 2.1 mm, 1.7 µm) by using mobile phase composed of aqueous phase A (0.1% formic acid) and organic phase B (acetonitrile: methanol, 80: 20 % v/v) in a gradient mode

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(Tmin/B; T0/10; T1.5/10;T3/50; T8.5/80; T9.5/80; T11/10; T12 /10). The flow rate, injection volume, and detection wavelength were 0.3 mL/min, 1µL and 261 nm, respectively. The same method was used for LC-HRMS studies.

Degradation behavior of REG

The degradation behavior of REG was monitored using UPLC-PDA and UPLC–MS under various stress degradation conditions. Optimum degradation was observed under all stress degradation conditions except photolysis and thermal degradation, where the drug was found to be stable. A total of five DPs (DP1 to DP5) were formed in hydrolysis (acid, base and neutral) and oxidative stress conditions (Fig. 1). Among all the DPs, two major degradation products (DP1 and DP2) were observed as common degradation products in all the hydrolysis (acid, base and neutral) conditions. The degradation product DP3 was formed under both acid hydrolysis and oxidation stress conditions. The drug was found to be more stable even after subjecting to thermal degradation at 100 °C for 15 days. The overlay chromatograms of all stress degradation samples of REG are given in Fig.1

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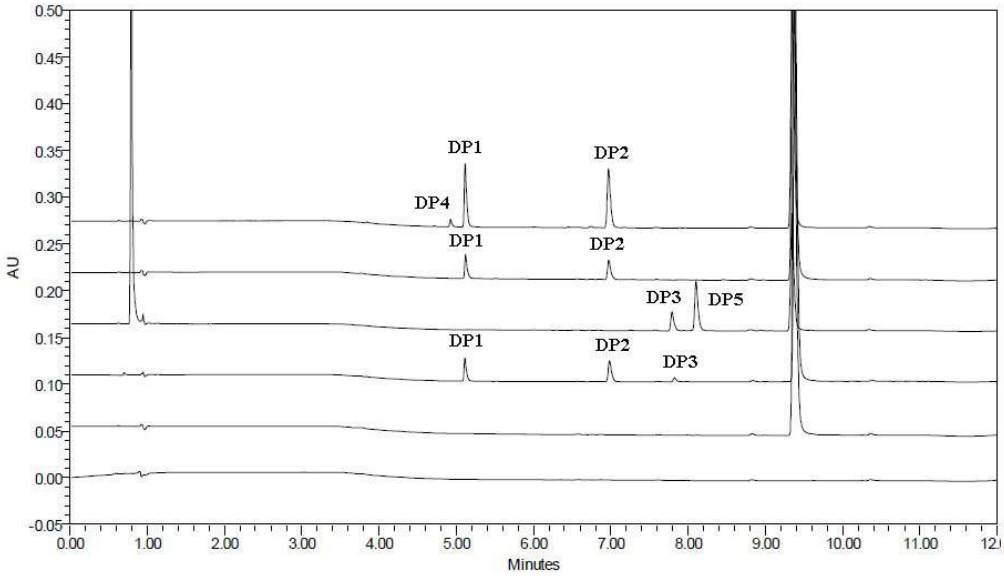

Fig. 1. Overlaid chromatograms of (a) Blank (ACN: water, 80: 20, %v/v), (b) standard (unstressed) of REG, (c) acid degradation of REG, (d) oxidative degradation of REG, (e) neutral degradation of REG, (f) base degradation of REG.

Characterization of REG and its DPs by LC–MS/MS

REG and all the DPs (DP1-5) were well separated by LC and they show abundant protonated molecular ions ($[M+H]^+$) in ESI positive ionization mode. The ESI-MS/MS spectra of all the five degradation products are shown in Fig. 2, and their elemental compositions and accurate masses are given in Table S1 (see the ESI, Electronic Supplementary Information).

MS/MS of $[M+H]^+$ of REG (*m/z* 483)

To understand the fragmentation pathway of protonated REG (*m/z* 483), its MS/MS spectrum was examined. The spectrum showed abundant product ions at *m/z* 465, *m/z* 443, *m/z* 424, and *m/z* 404. Other abundant product ions were observed at *m/z* 288, *m/z* 270, *m/z* 262, *m/z* 244, *m/z* 229, *m/z* 201 and *m/z* 193.

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The ion at m/z 288 can be formed due to cleavage of carbamide bond between chlorotrifluoromethyl benzene ring and rest of the moiety of REG. The elemental compositions of all above fragment ions (Scheme 1) have been confirmed by accurate mass measurements (Table S1).

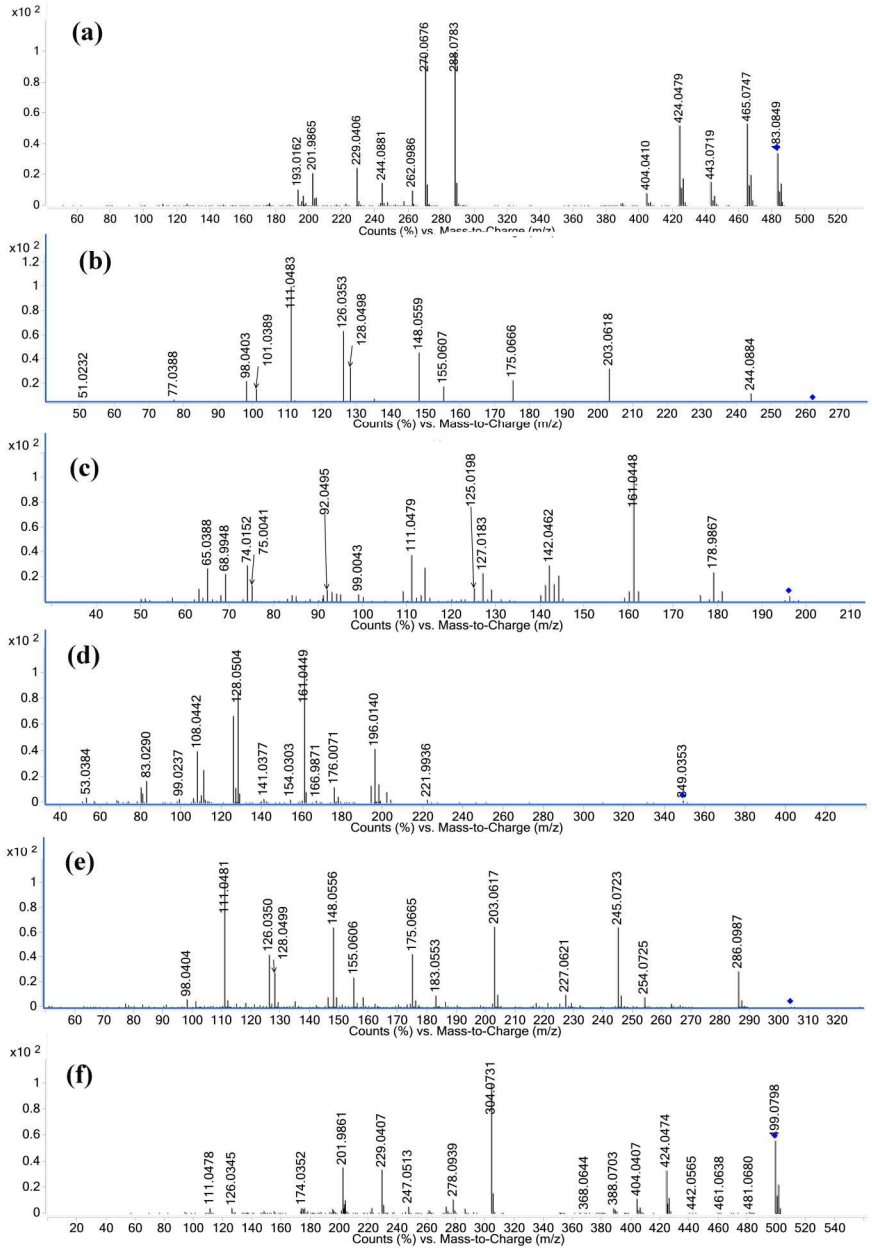

Fig. 2. LC-ESI/MS/MS spectra of $[M+H]^+$ ions of (a) REG, (b) DP1, (c) DP2, (d) DP3, (e) DP4, (f) DP5

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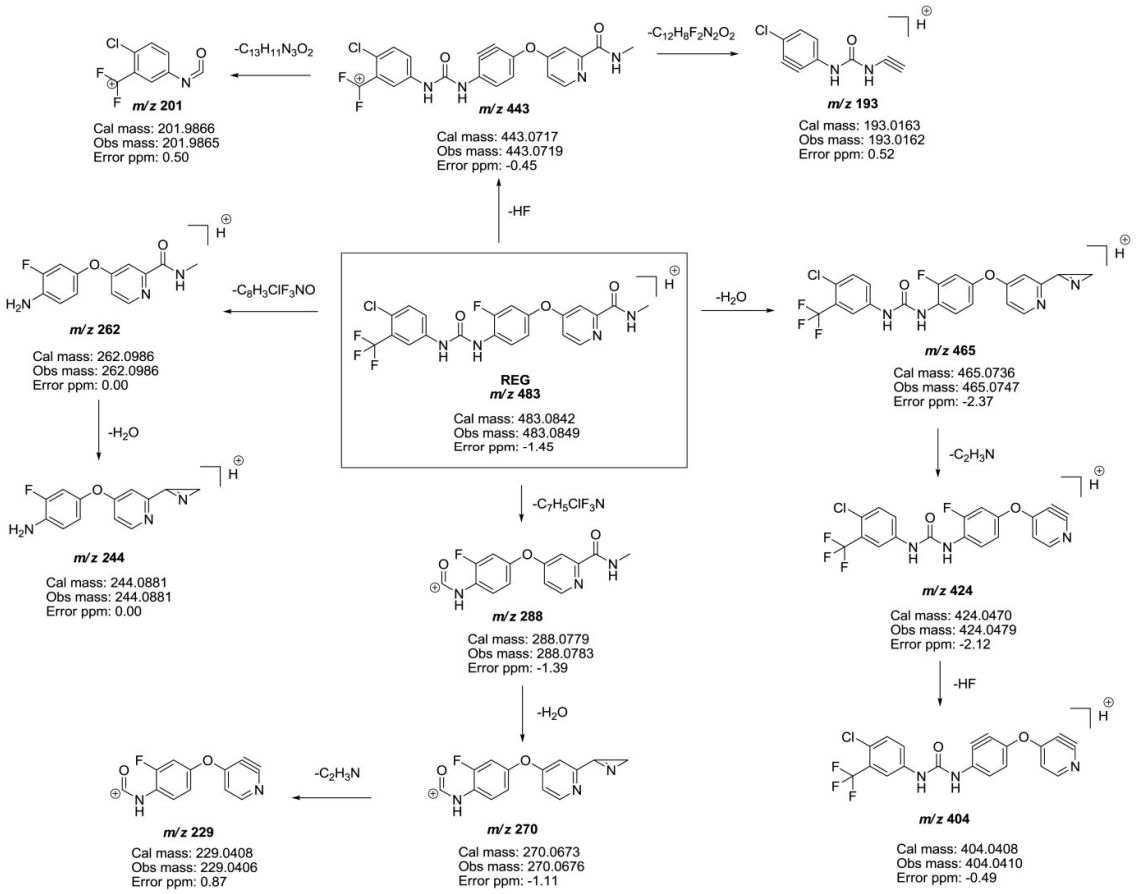

Scheme 1. Proposed fragmentation pathway of protonated **REG**

MS/MS of $[M+H]^+$ of DP1 (*m/z* 262)

The MS/MS spectrum of $[M+H]^+$ ions (*m/z* 262) of DP1 (Rt= 5.1 min) shows the product ions of *m/z* 244, *m/z* 203, *m/z* 175, *m/z* 155, *m/z* 148, *m/z* 128, *m/z* 126, *m/z* 111, *m/z* 101, *m/z* 98, *m/z* 77 and *m/z* 51 (Fig. 2b). It was observed that the $[M+H]^+$ of REG gives the ion at *m/z* 262 with same elemental composition that of DP1 (Scheme 2). However, the absence of abundant product ions at *m/z* 175, 155 and 148 in the MS/MS of protonated REG clearly points to structural

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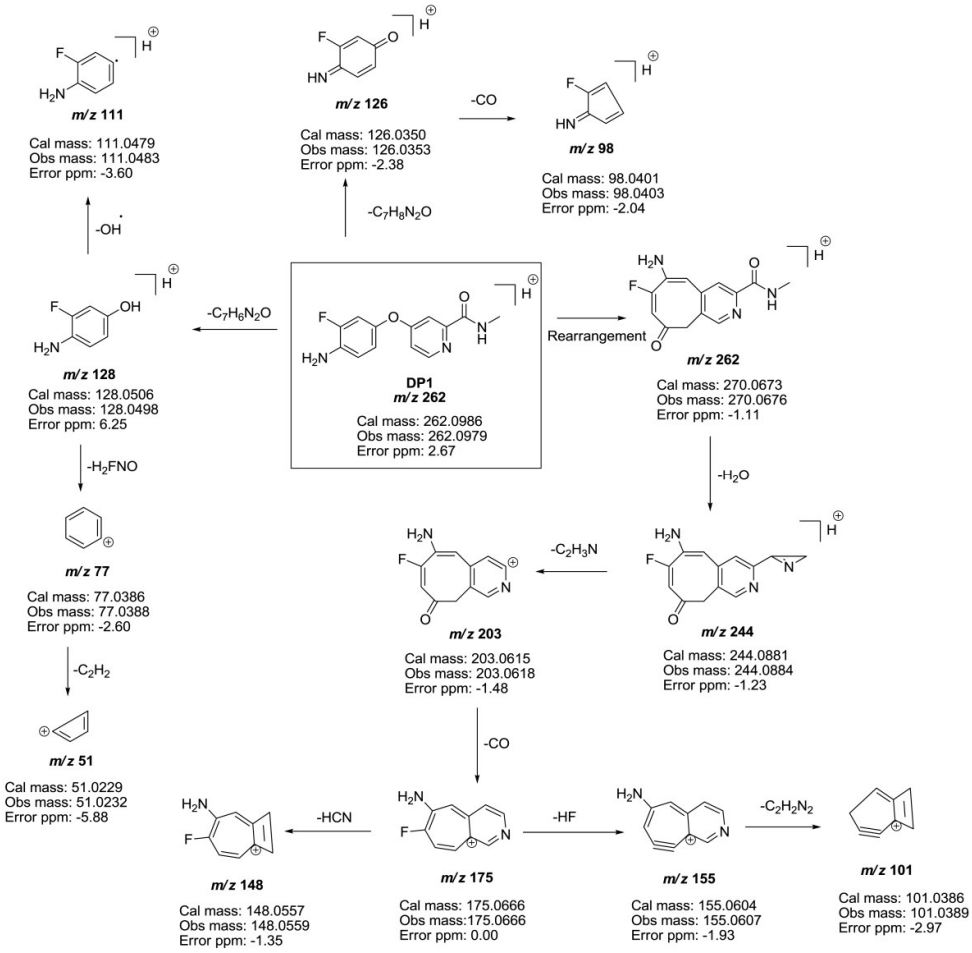
changes in DP1. Thus the formation of these product ions from DP1 can be attributed to rearrangement reactions following an *ortho*-effect in 4-phenoxypyridine ring moiety of DP1 (Scheme 3) during collision-induced dissociation (CID). This type of rearrangement reaction was reported in aromatic ethers, sulphides, and sulphoxides under EI conditions.[24,25] Based on the above data, the structure of DP1 could be proposed as 4-(4-amino-3-fluorophenoxy)-N-methylpicolinamide.



Scheme 2. Proposed fragmentation pathway of protonated **DP1**

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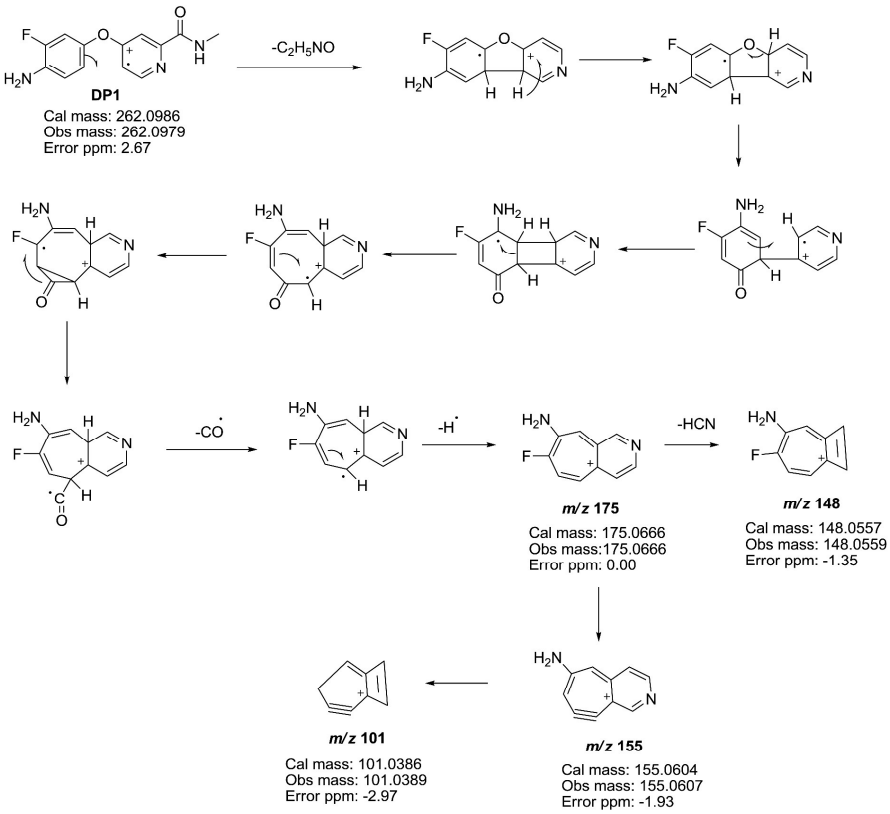

Scheme 3. Proposed reaction pathway for the formation of rearranged fragment ions (*m/z* 175, 155, 148 and 101) in DP1 and DP3

MS/MS of $[M+H]^+$ of DP2 (*m/z* 196)

The accurate mass of $[M+H]^+$ of DP2 (Rt= 6.9 min) was determined to be *m/z* 196.0138 (Fig. 2(c)) corresponding to an elemental composition of $C_7H_6ClF_3N^+$. The MS/MS spectrum of protonated DP2 is shown in Fig. 2(c). Scheme 4 includes the structure of DP2 and its product ions at *m/z* 178, *m/z* 176, *m/z* 161, *m/z* 142, *m/z* 127, *m/z* 125, *m/z* 111, *m/z* 99, *m/z* 92, *m/z* 75, *m/z* 74, *m/z* 68 and *m/z* 65. The diagnostic fragment ions at *m/z* 178, 161, 127 and 68 can be formed from *m/z* 196 by the loss of NH_3, Cl, CF_3 and C_6H_6ClN, respectively. Formation

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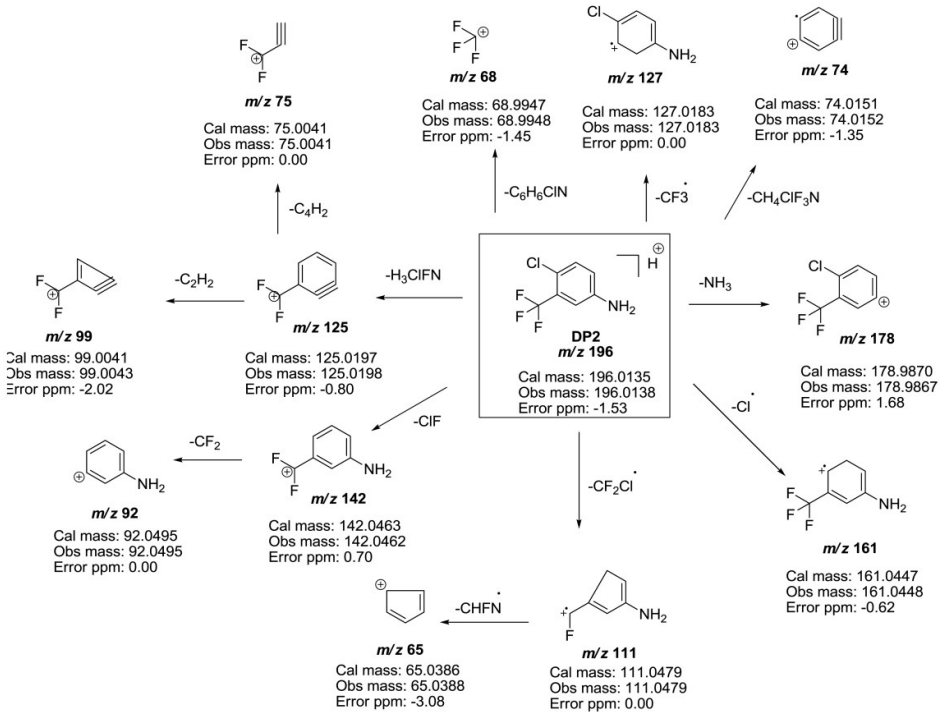
of radical fragment ions at *m/z* 161, 127, 111 and 74 involve homolytic bond cleavage during CIDs,[26] which is generally observed in EI-MS, are less common among CIDs under ESI and APCI conditions.[27] The characteristic product ions at *m/z* 127 and *m/z* 68 indicate that 4-chloroaniline and fluoroform moieties are intact in the structure of degradation product DP2. Based on fragmentation of protonated DP2 and accurate mass measurements, the structure of DP 2 could be proposed as 4-chloro-3-(trifluoromethyl)aniline.



Scheme 4. Proposed fragmentation pathway of protonated **DP2**

MS/MS of $[M+H]^+$ of DP3 (*m/z* 349)

The MS/MS spectrum (Fig. 2(d)) of protonated DP 3 (Rt 7.8 min, *m/z* 349) shows the product ions at *m/z* 221, *m/z* 196, *m/z* 176, *m/z* 166, *m/z* 161, *m/z* 154,

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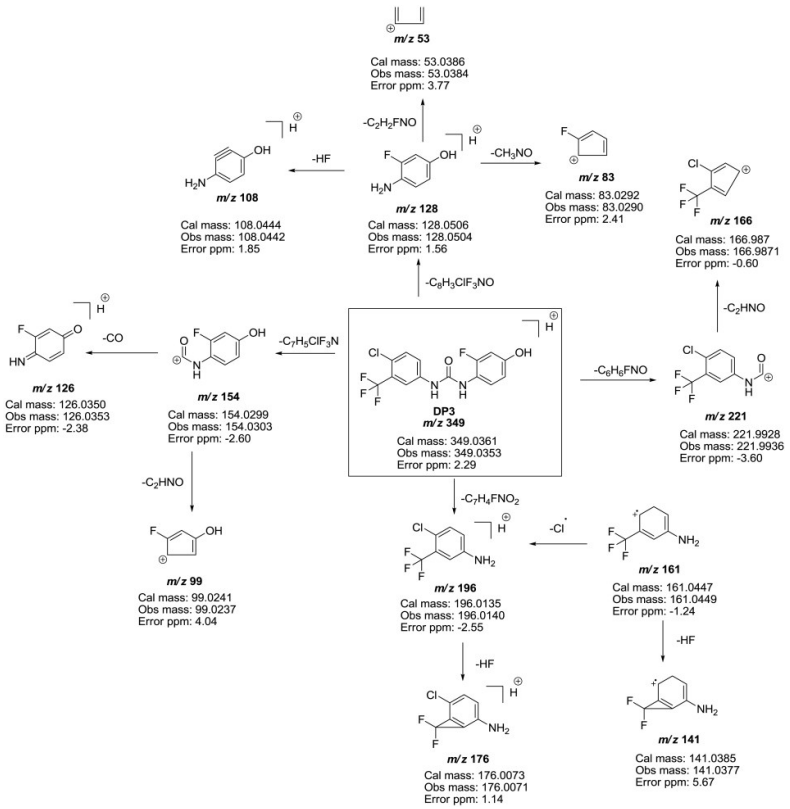
m/z 141, *m/z* 128, *m/z* 126, *m/z* 108, *m/z* 99, *m/z* 83 and *m/z* 53. The generation of intense ions at *m/z* 196, 176 and 161 indicates the presence of 4-chloro-3-(trifluoromethyl) aniline, whereas ions at *m/z* 128, 108 and 83 represents 4-amino-3-fluorophenol moieties are intact in the DP3. The formation of product ions at *m/z* 221 and *m/z* 154 displayed urea bond linking between 1-chloro-2-(trifluoromethyl)benzene and 3-fluorophenol rings. Based on the fragmentation pattern (Scheme 5), DP3 was identified as 1-(4-chloro-3-(trifluoromethyl)phenyl)-3-(2-fluoro-4-hydroxyphenyl)urea. The elemental compositions have been confirmed for $[M+H]^+$ of DP3 and its fragment ions by accurate mass measurements.



Scheme 5. Proposed fragmentation pathway of protonated **DP3**

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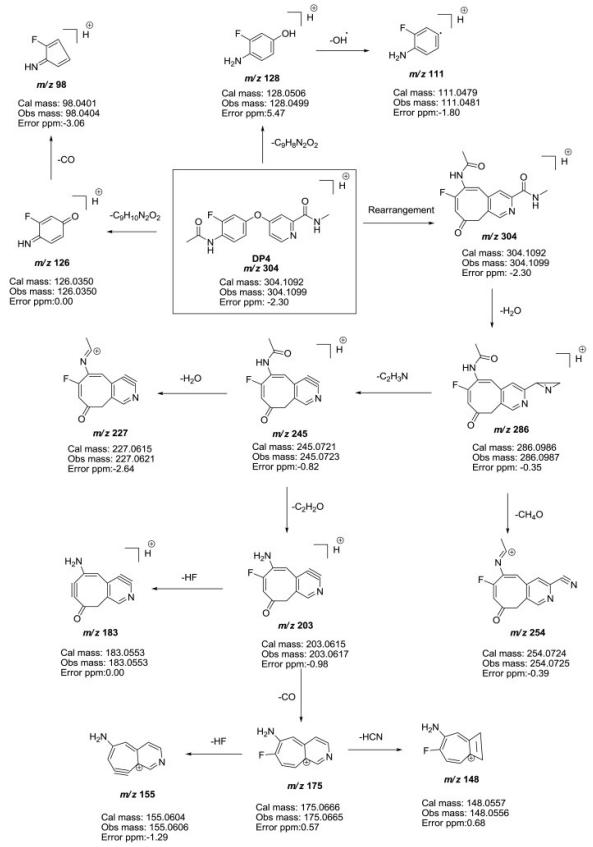
MS/MS of $[M+H]^+$ of DP4 (*m/z* 304)

The MS/MS spectrum of protonated DP4((Rt= 4.9 min, *m/z* 304 $C_{15}H_{15}FN_3O_3^+$), displayed product ions at *m/z* 286, *m/z* 245, *m/z* 227, *m/z* 203, *m/z* 183, *m/z* 175, *m/z* 155, *m/z* 148, *m/z* 128, *m/z* 126, *m/z* 111 and *m/z* 98. The common fragment ions at *m/z* 175, 155, 148 (Scheme 3), 126 and 111 were generated from 4-amino-5-fluoro-2-(pyridin-4-yl)phenol moiety and it was intact in both the DPs (DP1 and DP4). Based on the fragmentation pattern (Scheme 6) and accurate measurements, DP4 was identified as 4-(4-acetamido-3-fluorophenoxy)-*N*-methylpicolinamide.



Scheme 6. Proposed fragmentation pathway of protonated **DP4**

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MS/MS of $[M+H]^+$ of DP5 (*m/z* 499)

The degradation product, DP5 (Rt = 8.1min) was formed under oxidation stress condition. The mass difference between the protonated drug (*m/z* 483) and DP5 (*m/z* 499) is 16 Da, which indicates an addition of oxygen from hydrogen peroxide to REG as hydroxylated form or N-oxide of the drug. The MS/MS spectrum of DP5 displayed product ions at *m/z* 481, *m/z* 461, *m/z* 442, *m/z* 424, *m/z* 404, *m/z* 388, *m/z* 368, *m/z* 304, *m/z* 278, *m/z* 247, *m/z* 229, *m/z* 201, *m/z* 174, *m/z* 126 and *m/z* 111 (Scheme 7). The product ion at *m/z* 424 indicated the presence of 1-(4-chloro-3-(trifluoromethyl)phenyl)-3-(2-fluoro-4-(pyridin-4-yloxy)phenyl)urea moiety which is similar to that of the drug (Scheme 1). However, the presence of product ions at *m/z* 304 and 278, with a mass of 16 Da more than the corresponding fragment ions of the drug (*m/z* 288 and 262), showed that a structural change occurred in 4-(4-amino-3-fluorophenoxy)-N-methylpicolinamide moiety. The product ions at *m/z* 278 and 247 suggested that hydroxylation had taken place presumably on the picolinamide ring. Atmospheric pressure chemical ionization (APCI)-MS experiment was performed to distinguish between N-oxide and hydroxylated product. It was observed that there is no instant loss of OH radical, which is generally observed in the case of N-oxide by APCI-MS experiment. It was reported that N-oxides are thermally labile and undergo deoxygenation by thermal decomposition (thermal energy activation in the vaporizer of the APCI source) in the APCI.[28,29]

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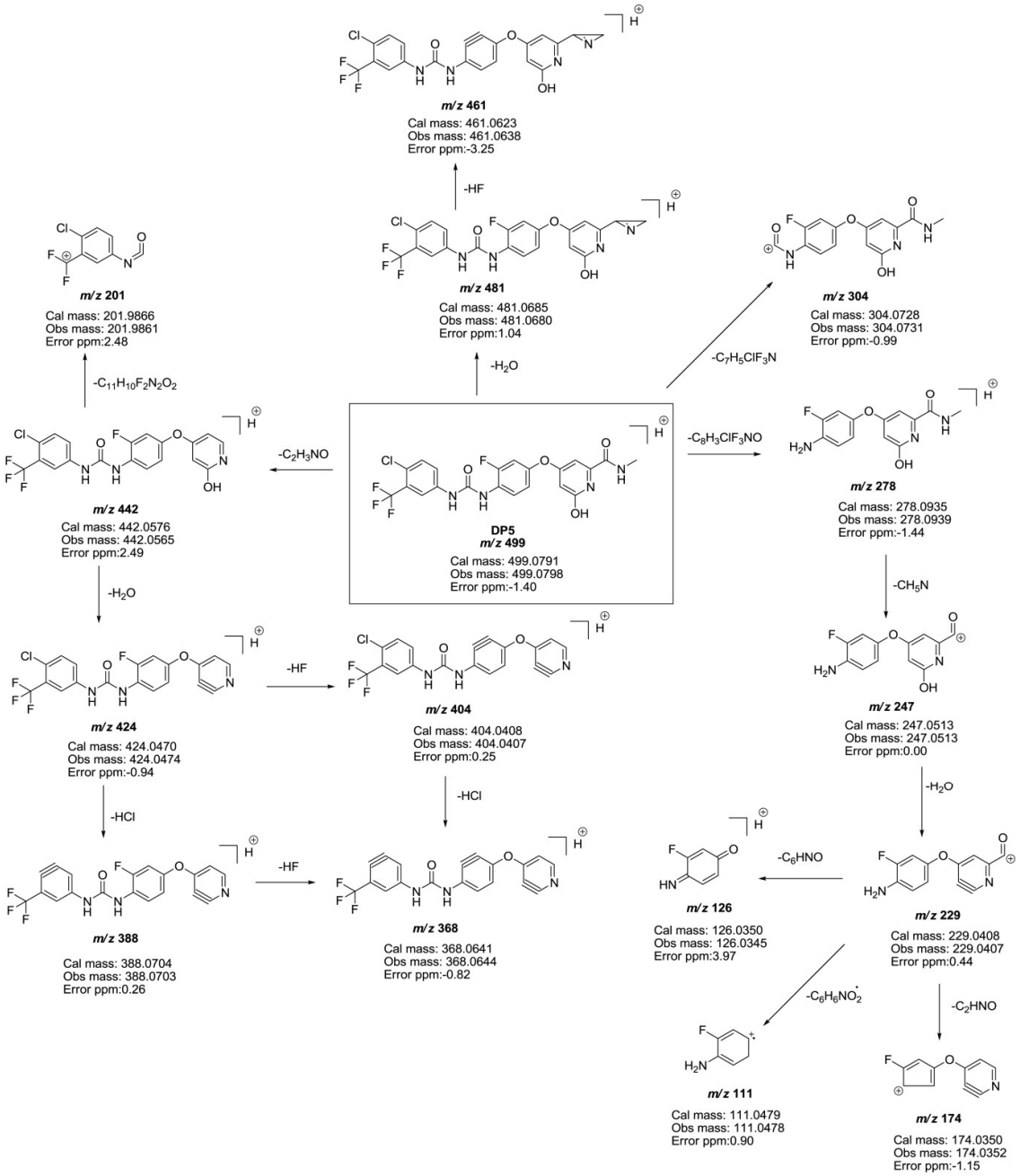

Scheme 7. Proposed fragmentation pathway of protonated **DP5**

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The structure of degradation product DP1 was similar to one of the key intermediate while DP2 mimic the starting material in the synthesis of REG [15,16] and DP3 matched with regorafenib urea impurity (CAS No 1333390-56-9). The DP5 (hydroxylated DP) did not mimic with any of the process impurities or metabolites but closely similar to regorafenib-N-Oxide [19] (M2 Metabolite, CAS No 835621-11-9) and hydroxyregorafenib (M3 Metabolite).

NMR structural characterization studies

The degradation product DP1 and REG (Fig. 3) were subjected to 1D NMR (^{1}H, ^{13}C and DEPT) and 2D NMR (COSY, ROESY, HSQC and HMBC) experiments to verify the proposed structure of DP1. The data are compiled in Table S2 (see the ESI). ^{1}H and ^{13}C NMR experiments in DMSO-d_6 reveal that REG contained 15 protons and 21 carbons while DP1 contained 12 protons and 13 carbons. Based on DEPT-90, DEPT-135, and ^{13}C NMR experiments, DP1 has 1-CH3 (primary), 6-CH (tertiary) and 6-C (quaternary) carbons, while REG has 1-CH3 (primary), 9-CH (tertiary) and 10-C (quaternary) carbons. In ^{1}H NMR spectrum of DP1, the protons at H-12, H-15, H-16, and H-17 were shielded to 6.99, 6.84, 6.77 and 5.20 ppm when compared with REG this revealed that absence of 1-(4-chloro-3-(trifluoromethyl)phenyl)urea moiety which is present in the parent molecule (Fig. 3).

In addition, ^{13}C NMR spectra were consistent with above-mentioned observations, the carbon C-14 was shifted to deshielding as 142.2 ppm from

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124.9 ppm because of –NHCONH group attached to C-14 was replaced by the simple NH_2 group. The structure of DP1 was further confirmed by COSY and ROESY experiments where all the correlations are in accordance with the proposed structure.

Other 2D techniques such as HSQC and HMBC were also performed to verify the structure of DP1. The HSQC spectra of DP1 and REG are shown in Fig. 4. HSQC spectra of DP1 shows the presence of correlation of CH_3 (9) and CH (2,4,5,12,15,16) and absence of correlations of CH (21, 24, 25) which were observed in REG HSQC spectra (Fig. 4). In addition, HMBC spectra of DP1 and REG were used to verify the correlations between the protons and carbons in the structural framework, which were ≥2 bonds apart. Moreover, notable correlations observed in HMBC spectra also helped to assign chemical shift values corresponding to protons and carbons. Based on the observations from 1D NMR (^{1}H, ^{13}C, DEPT-90, DEPT-135) and 2D NMR (COSY, ROESY, HSQC, and HMBC) spectra, the structure of DP1 was identified as 4-(4-amino-3-fluorophenoxy)-N-methylpicolinamide (Fig. 3).

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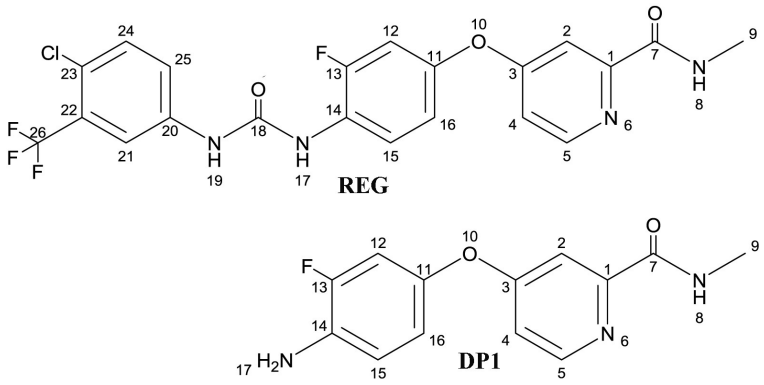

Fig. 3. Schematic diagram of chemical structures of REG and DP1 with number depiction

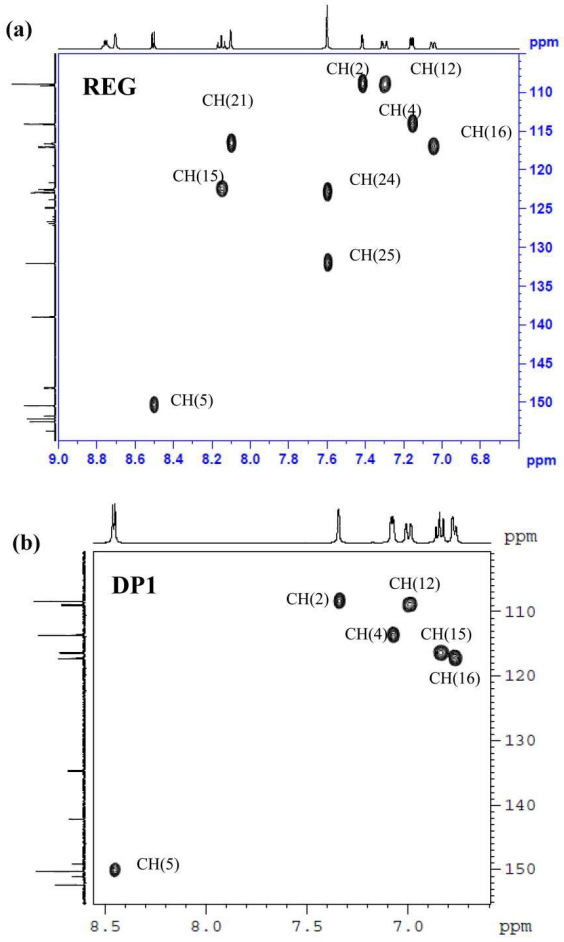

Fig. 4. HSQC spectra of REG and DP1 (500 MHz, DMSO-d_6)

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Mechanistic explanation of formation of degradation products (DP1-DP5)

The formation of degradation products (DP1-DP5) was shown in Scheme 8. DP1 and DP2 were formed as major degradation products under acid, base, and neutral hydrolytic condition because of susceptibility of urea bond to all hydrolytic (acid, base and neutral) conditions. DP1 and DP2 were formed by hydrolytic cleavage of urea bond. The degradation product DP3 was formed by cleavage of diphenyl ether linkage of REG under acid hydrolysis and oxidative stress conditions. Formation of DP4 can be explained by hydrolysis of urea bond to form carbamic (R-NHCOOH) acid followed by its conversion to acetamide (R-NHCOCH$_3$). The degradation product DP5 was formed only under oxidative stress condition and it can be explained by an addition of hydroxyl group to N-methylpicolinamide ring of REG.

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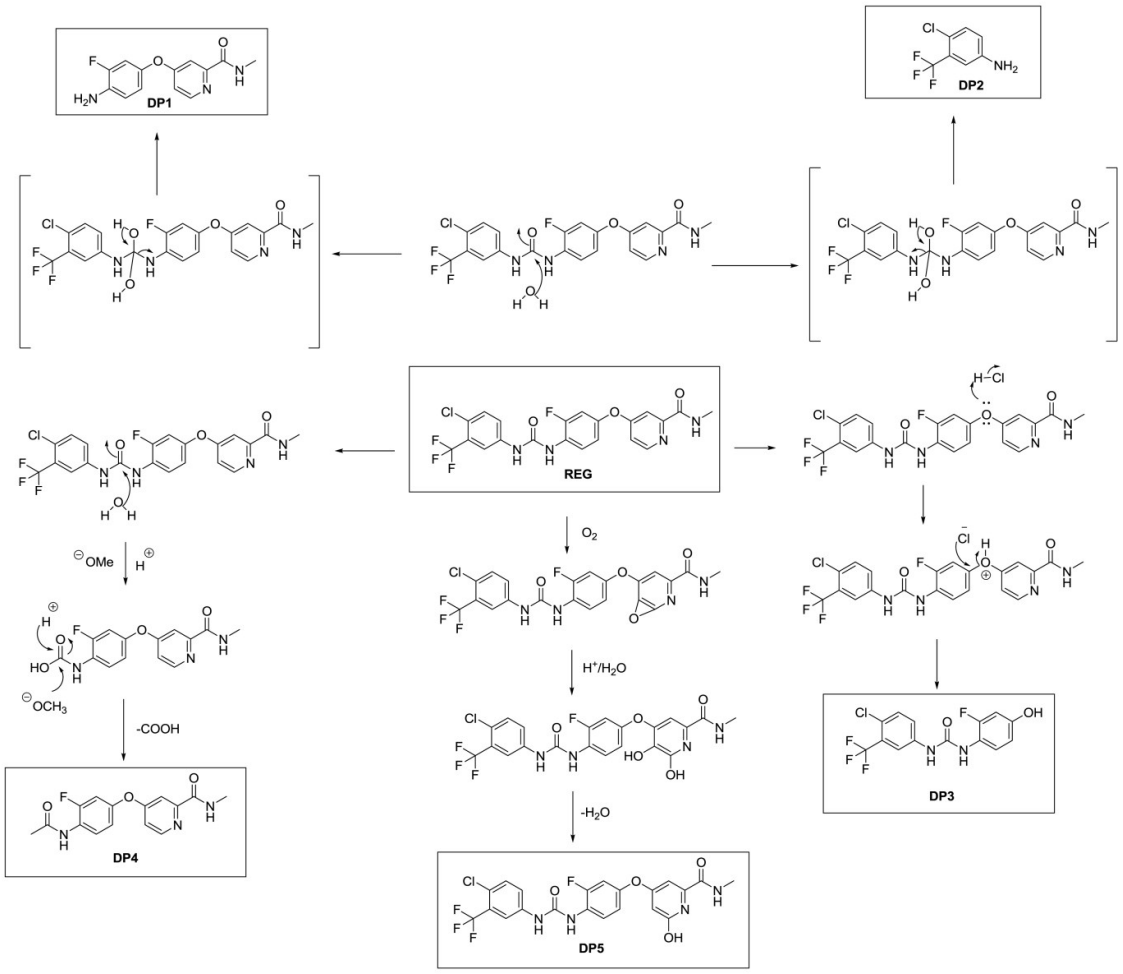

Scheme 8. Probable mechanisms of formation of degradation products **(DP1-DP5)**

IR structural characterization studies

The major differences in the functional groups and similarities between REG and DP1were evaluated by comparing the IR spectra of DP1 with that of REG. The major differences found at 3388.92, 3349.84 and 1718.59 cm^{-1} (IR spectra of REG) corresponding to NH and C=O stretch of urea bond of REG which is absent in the DP1 spectra. The peaks at 3394.96 and 1567.74 cm^{-1} (IR spectra of DP1) represented primary amine ($-NH_2$) group which is absent in the spectra of REG. Group of peaks at 1140.05, 1128.94 and 1106.97 cm^{-1} (IR spectra of

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REG) indicates the C-F bond in CF_3 functional group while sharp peak was observed at 1140.52 cm^{-1} (IR spectra of DP1) represent the C-F bond. The C-Cl bond peak was observed at 650.69 cm^{-1} (IR spectra of REG) which are absent in the spectrum of DP1.

The IR spectra of DP1 compared with REG was utilized to find the similarities at 1655.38 cm^{-1} corresponding to C=O stretch of amide group which is present at 1666.09 cm^{-1} in the spectra of DP1. The peaks at 1595.21and 1539.27 cm^{-1} (IR spectra of REG) indicated NH bending vibration of mono-substituted amide bond while in the spectra of DP1 it was observed at 1535.88 cm^{-1}. Other peaks can observe at 1504.48 cm^{-1} (IR spectra of REG) and 1507.87 cm^{-1} (IR spectra of DP1) represented aromatic CH bond. The peaks were observed at 870.62, 835.41, 742.99 cm^{-1} (IR spectrum of REG) and 890.08, 848.32, 803.03 cm^{-1} (IR spectra of DP1) indicates the 1,2 disubstitution or 1,3 disubstitution, 1,4 disubstitution and 1,2,4 trisubstitution respectively.

In silico toxicity prediction of REG and its DPs

The results of toxicity prediction by TOPKAT are shown in Table S3 (see the ESI). Assessment of these results shows similarity in the toxicity profile of the drug and its degradation products. But some of the animal models show the variations for them. DP2 shows a high probability for rat male NTP (National Toxicology Program). All compounds show very high and similar toxicity profile for mouse male NTP, mouse female NTP and ocular irritancy mild *vs* moderate severe (with the exception of DP2 shows non-toxic profile). Also for

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rat male FDA (Food and Drug Administration) single *vs* multiple prediction DP4 exceptionally shows non-toxic profile. In a case of skin sensitization weak *vs* strong model, DP2 and DP3 remarkably shows non-toxic endpoints whereas other compounds show a high probability of toxicity. Skin sensitization none *vs* sensitizer model shows extremely high toxicity endpoint for DP2. The DP1 shows extreme high toxicity probability for mouse female FDA none *vs* carcinogen.

Variations have also been observed in the form low toxicity profile. DP1, DP2, and DP4 show low probabilities for rat male FDA none *vs* carcinogen and skin irritancy none *vs* irritant model. DP5 has shown in weight of evidence rodent carcinogenicity model. DP2 and DP3 have been found to be least toxic for developmental toxicity potential. However DP1 and parent drug show intermediate probability for mouse male FDA none *vs* carcinogen. DP4 shows for mouse female FDA none *vs* carcinogen.

The qualitative results from DEREK software are shown in Table S4 (see the ESI). Qualitative toxicity profiling of regorafenib and its DPs shows a presence of carcinogenicity, skin sensitization and peroxizome proliferation toxicity end points. The parent drug regorafenib, DP1, DP4 and DP5 have shown carcinogenicity and peroxizome proliferation toxic end points in mouse and rat models. These toxicity end points have been observed due to a presence of alkylaryl or bisaryl carboxylic acid or precursor moiety. DP1and DP2 have shown skin sensitization toxicity end point due to the presence of aromatic

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primary or secondary amine. This DEREK toxicity profile for DP1 and DP2 further confirms the results obtained using TOPKAT. DP3 didn't show any toxicity profile. Both studies (TOPKAT and DEREK) further confirm the carcinogenicity toxicity profile of DP1, DP4, and DP5.

Conclusion

In conclusion, a formation of all DPs (DP1-DP5) from REG under hydrolysis (acidic, basic and neutral) and oxidation stress condition was discussed. The structure of DPs (DP2-DP5) was elucidated by using LC–MS/MS and the major degradation product (DP1) was identified by using LC–MS/MS, multidimensional NMR and FTIR analytical techniques. The LC-MS/MS and NMR data supported the observed rearrangement reactions in DP1 and DP4. Additionally *in silico* toxicity data were predicted for degradation products by using DEREK and TOPKAT software tools. The parent drug (REG) and DPs (DP1, DP4, and DP5) shows peroxizome proliferation toxic end points and carcinogenicity in mouse and rat models. DP1and DP2 have shown skin sensitization whereas DP3 has not shown any toxicity profile.

Conflicts of interest

There are no conflicts of interest to declare

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