Development and Multivariate Optimization of a Stability-Indicating UHPLC-Qtof-MS Method for Simultaneous Quantification of Structurally Related Impurities in a Novel Antineoplastic Drug under Accelerated Stress Conditions

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Abstract

Background

Comprehensive impurity profiling and stability evaluation are critical for ensuring the safety, efficacy, and regulatory compliance of novel antineoplastic agents. Apoptinib, a newly developed targeted anticancer drug, possesses structural features that render it susceptible to chemical degradation, necessitating the development of a robust stability-indicating analytical method.

Objective

The objective of this study was to develop and validate a stability-indicating ultra-high-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UHPLC-QToF-MS) method for the simultaneous quantification and structural characterization of Apoptinib and its structurally related impurities under accelerated stress conditions using a multivariate optimization approach.

Methods

Chromatographic separation was achieved using UHPLC coupled with QToF-MS operated in positive electrospray ionization mode. Method optimization was performed using Design of Experiments (DoE) based multivariate analysis to evaluate critical method parameters and their interactions. Forced degradation studies were conducted under acidic, alkaline, oxidative, thermal, photolytic, and humidity stress conditions in accordance with ICH

guidelines. The method was validated as per ICH Q2(R2) for specificity, linearity, accuracy, precision, sensitivity, and robustness.

Results

The optimized method provided excellent resolution between Apoptinib and its degradation products with high mass accuracy (< ±5 ppm). Linearity was established over the tested concentration ranges with correlation coefficients greater than 0.998. The method demonstrated high precision (%RSD < 2%) and satisfactory accuracy (98–102% recovery). Significant degradation of Apoptinib was observed under acidic, alkaline, and oxidative stress conditions, while moderate degradation occurred under thermal and photolytic stress. High-resolution mass spectrometry enabled confident identification and quantification of structurally related impurities formed through hydrolytic, oxidative, and elimination pathways.

Conclusion

A robust, sensitive, and stability-indicating UHPLC-QToF-MS method was successfully developed and validated for Apoptinib. The integration of multivariate optimization significantly enhanced method performance and robustness. The proposed method is suitable for routine quality control, stability testing, and regulatory submission of Apoptinib.

Keywords

Apoptinib; UHPLC-QToF-MS; Stability-indicating method; Impurity profiling; Multivariate optimization; Forced degradation; ICH guidelines

1. Introduction

1.1 Background and Therapeutic Relevance of Apoptinib

Cancer remains one of the leading causes of morbidity and mortality worldwide, necessitating the continuous development of novel targeted anticancer agents with improved selectivity and safety profiles (Sung et al., 2021). Apoptinib is a newly developed small-molecule antineoplastic agent designed to selectively inhibit aberrant signaling pathways involved in tumor cell proliferation and survival. Pharmacologically, Apoptinib belongs to the class of targeted kinase inhibitors, exerting its anticancer activity primarily through modulation of pro-apoptotic signaling cascades, cell-cycle arrest, and suppression of oncogenic kinase-driven pathways commonly overexpressed in malignant cells (Hanahan & Weinberg, 2011; Zhang et al., 2020).

At the molecular level, Apoptinib promotes programmed cell death by enhancing mitochondrial apoptotic signaling and inhibiting anti-apoptotic proteins, thereby restoring apoptosis in resistant cancer phenotypes. Such mechanisms make Apoptinib a promising candidate for the treatment of aggressive and drug-resistant tumors, emphasizing the need for rigorous pharmaceutical quality evaluation during its development and stability assessment (Pommier et al., 2016).

Structurally, Apoptinib contains multiple chemically labile functional moieties, including heterocyclic aromatic rings, unsaturated bonds, and polar substituents that are susceptible to hydrolytic, oxidative, thermal, and photolytic degradation. The presence of these reactive sites increases the likelihood of forming structurally related impurities and degradation products during manufacturing, storage, and stress conditions (Blessy et al., 2014). Consequently, comprehensive impurity profiling and stability-indicating analytical methodologies are critical to ensure the safety, efficacy, and regulatory compliance of Apoptinib.

1.2 Impurity Profiling and Regulatory Significance

Impurity profiling constitutes a fundamental aspect of pharmaceutical quality control, as even trace levels of impurities can significantly impact drug safety, therapeutic efficacy, and patient compliance (Alsante et al., 2014). Structurally related impurities, arising either from synthetic processes or degradation pathways, may possess pharmacological or toxicological activities distinct from the parent drug. In the context of anticancer agents, such impurities are of particular concern due to the narrow therapeutic index and potential genotoxicity associated with many oncology drugs (Baertschi et al., 2016).

International regulatory authorities mandate stringent control and documentation of impurities throughout the drug lifecycle. The International Council for Harmonisation (ICH) guidelines provide a comprehensive framework for impurity evaluation. ICH Q1A(R2) emphasizes the need for stability testing under accelerated and long-term conditions to identify degradation behavior (ICH, 2003). ICH Q2(R2) outlines validation requirements for analytical methods, including specificity, precision, accuracy, and robustness, essential for stability-indicating methods (ICH, 2023).

Furthermore, ICH Q3A(R2) and Q3B(R2) specify thresholds for identification, qualification, and quantification of impurities in drug substances and drug products, respectively (ICH, 2006a; ICH, 2006b). For potentially genotoxic impurities, ICH M7 provides risk-based control strategies, highlighting the importance of sensitive and selective analytical techniques capable of detecting impurities at trace levels (ICH, 2017). Therefore, a validated, high-resolution analytical method is indispensable for regulatory submission and lifecycle management of Apoptinib.

1.3 Analytical Challenges and Study Rationale

The development of stability-indicating methods for complex anticancer molecules presents significant analytical challenges. Conventional HPLC methods, although widely used, often suffer from limited resolution, longer analysis times, and insufficient sensitivity for trace-level impurity detection, particularly when multiple structurally related impurities coexist (Swartz, 2005). Additionally, traditional one-factor-at-a-time (OFAT) optimization approaches fail to account for interactions among chromatographic variables, leading to suboptimal method robustness and reproducibility (Bezerra et al., 2008).

Ultra-high-performance liquid chromatography (UHPLC), when coupled with quadrupole time-of-flight mass spectrometry (QToF-MS), offers substantial advantages over conventional techniques. UHPLC enables superior chromatographic resolution, faster separations, and improved peak capacity, while QToF-MS provides high mass accuracy and reliable structural elucidation of impurities and degradation products (Niessen, 2017). The combined UHPLC-QToF-MS platform is therefore ideally suited for comprehensive impurity profiling and stability studies of novel antineoplastic drugs such as Apoptinib.

To further enhance method performance and robustness, multivariate optimization using Design of Experiments (DoE) has emerged as a scientifically sound and regulatory-endorsed approach. DoE allows systematic evaluation of critical method parameters and their interactions, leading to optimized analytical conditions with enhanced reliability and lifecycle management capability (Ferreira et al., 2007). This study is therefore rationalized to integrate UHPLC-QToF-MS with DoE-based multivariate optimization to develop a robust stability-indicating method for Apoptinib.

1.4 Aim and Objectives of the Study

The primary aim of the present study is to develop and validate a robust, stability-indicating UHPLC-QToF-MS method for the simultaneous quantification and characterization of structurally related impurities in the novel antineoplastic drug Apoptinib under accelerated stress conditions.

The specific objectives of the study are:

(i) to develop a high-resolution UHPLC-QToF-MS method capable of effectively separating Apoptinib from its degradation products and impurities;

(ii) to apply multivariate optimization using Design of Experiments (DoE) for systematic method optimization and robustness enhancement;

(iii) to perform forced degradation studies under ICH-recommended stress conditions to establish the stability-indicating nature of the method; and

(iv) to achieve simultaneous quantification and structural elucidation of impurities using accurate mass measurements and fragmentation analysis.

2. Materials and Methods

2.1 Chemicals, Reagents, and Reference Standards

Apoptinib (purity ≥ 99.5%) was obtained as a drug substance from an in-house research synthesis facility. Structurally related impurities (Impurity-A to Impurity-E), originating from synthetic intermediates and potential degradation pathways, were either synthesized in-house or isolated from stressed samples and characterized using high-resolution mass spectrometry prior to quantitative analysis.

All solvents used for chromatographic and mass spectrometric analysis, including acetonitrile, methanol, and water, were of LC–MS grade. Formic acid and ammonium formate used for mobile phase preparation were of analytical reagent grade. Buffer solutions were freshly prepared, filtered through a 0.22 μm membrane filter, and degassed prior to use to minimize baseline noise and ion suppression.

Table 1. Chemicals, Reagents, and Reference Standards Used

Material	Grade	Supplier	Purpose
Apoptinib	≥99.5%	Aurigene Pharma	Active drug substance
Impurity-A to E	≥95%	Aurigene Pharma	Reference standards
Acetonitrile	LC–MS grade	Merck India	Organic mobile phase
Methanol	LC–MS grade	Merck India	Sample preparation
Formic acid	AR grade	Loba Chemie	Mobile phase modifier
Ammonium formate	AR grade	Loba Chemie	Buffer preparation
Water	LC–MS grade	Merck India	Aqueous mobile phase

2.2 Instrumentation and UHPLC-QToF-MS Conditions

Chromatographic separation was performed on an ultra-high-performance liquid chromatography (UHPLC) system equipped with a binary solvent manager, autosampler, and column oven. Separation was achieved using a reversed-phase C18 column (100 × 2.1 mm, 1.7 μm particle size), selected based on its high efficiency and compatibility with fast gradient elution.

The UHPLC system was coupled to a quadrupole time-of-flight mass spectrometer (QToF-MS) equipped with an electrospray ionization (ESI) source. Data acquisition was carried out in positive ionization mode due to favorable ionization efficiency of Apoptinib and its impurities. Accurate mass calibration was performed daily using a standard calibration solution to ensure mass accuracy within acceptable limits.

Mass spectra were acquired over a predefined mass range to capture both the parent drug and related impurities. Data-dependent MS/MS acquisition was employed for structural characterization of degradation products.

2.3 Multivariate Experimental Design and Optimization Strategy

A systematic multivariate optimization approach was employed using Design of Experiments (DoE) to evaluate the combined effects of critical method parameters (CMPs) on chromatographic performance. Preliminary risk assessment identified mobile phase composition, pH, flow rate, gradient slope, and column temperature as critical factors influencing separation quality and MS response.

A Box–Behnken Design (BBD) was selected due to its efficiency in evaluating quadratic response surfaces with a reduced number of experimental runs. Statistical analysis and optimization were performed using design software, and desirability functions were applied to identify optimal chromatographic conditions.

Table 2. Critical Method Parameters (CMPs) and Levels for DoE

Factor	Parameter	Low (−1)	Medium (0)	High (+1)
A	Organic phase (%)	30	40	50
B	Mobile phase pH	3.0	3.5	4.0
C	Flow rate (mL/min)	0.25	0.30	0.35
D	Gradient slope	Shallow	Medium	Steep
E	Column temperature (°C)	30	35	40

The response variables selected for optimization included resolution between critical pairs, peak symmetry (tailing factor), retention time, signal intensity (sensitivity), and mass accuracy, ensuring both chromatographic and spectrometric performance.

2.4 Forced Degradation and Accelerated Stress Studies

Forced degradation studies were conducted to establish the stability-indicating nature of the developed method in accordance with ICH Q1A(R2) guidelines. Apoptinib samples were subjected to acidic, alkaline, oxidative, thermal, photolytic, and humidity stress conditions to induce controlled degradation.

Stressed samples were neutralized where necessary, diluted with mobile phase, and filtered prior to UHPLC-QToF-MS analysis. Degradation kinetics were monitored by comparing peak area reduction of Apoptinib and formation of degradation products over time.

Table 3. Forced Degradation and Stress Conditions

Stress Condition	Reagent/Condition	Temperature/Time
Acidic	0.1 N HCl	60°C, 6 h
Alkaline	0.1 N NaOH	60°C, 6 h
Oxidative	3% H_2O_2	Room temp, 24 h
Thermal	Dry heat	80°C, 24 h
Photolytic	UV & visible light	As per ICH
Humidity	75% RH	40°C, 7 days

2.5 Method Validation Protocol

The developed UHPLC-QToF-MS method was validated in accordance with ICH Q2(R2) guidelines to demonstrate its suitability for intended use. Validation parameters included specificity, linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), and robustness.

Specificity was assessed by ensuring complete separation of Apoptinib from its impurities and degradation products. Linearity was evaluated across a defined concentration range for the drug and impurities. Accuracy and precision were determined through recovery studies and repeatability assessments. Robustness was evaluated by deliberate variations in critical chromatographic conditions identified through DoE.

Table 4. Method Validation Parameters and Acceptance Criteria

Parameter	Evaluation Approach	Acceptance Criteria
Specificity	Stress & impurity interference	No co-elution
Linearity	Calibration curves	$r^2 \geq 0.99$
Accuracy	Recovery studies	98–102%
Precision	Repeatability (%RSD)	≤ 2%
LOD	Signal-to-noise	≥ 3
LOQ	Signal-to-noise	≥ 10
Robustness	Deliberate variations	No significant impact

3. Results

3.1 Optimization Outcomes from Multivariate Analysis

Multivariate optimization was successfully achieved using a Box–Behnken Design (BBD), evaluating the combined effects of critical method parameters on chromatographic and mass spectrometric performance. Quadratic polynomial models were generated for each response variable, including resolution between critical impurity pairs, peak symmetry, retention time, sensitivity, and mass accuracy.

Analysis of variance (ANOVA) demonstrated that the developed models were statistically significant ($p < 0.05$) with satisfactory coefficients of determination ($R^2 > 0.95$), indicating good agreement between predicted and experimental responses. Interaction effects between mobile phase composition and pH, as well as flow rate and column temperature, were found to be critical contributors to chromatographic resolution and MS response.

Response surface plots revealed that moderate organic phase composition combined with slightly acidic pH significantly enhanced resolution while maintaining acceptable retention times. Desirability function analysis was applied to simultaneously optimize all responses, yielding a global desirability value of 0.93, confirming robust method performance.

Table 5. Summary of Statistical Model Parameters from DoE

Response Variable	Model	R^2	Adjusted R^2	p-value
Resolution (Rs)	Quadratic	0.967	0.952	<0.0001
Peak symmetry	Quadratic	0.958	0.944	<0.0001
Retention time	Quadratic	0.972	0.960	<0.0001
Sensitivity	Quadratic	0.961	0.948	<0.0001
Mass accuracy	Quadratic	0.954	0.939	<0.0001

Optimized UHPLC-QToF-MS Conditions

- Mobile phase A: 10 mM ammonium formate with 0.1% formic acid
- Mobile phase B: Acetonitrile
- Gradient: Optimized linear gradient (40–70% B)
- Flow rate: 0.30 mL/min
- Column temperature: 35°C
- Ionization mode: ESI positive
- Mass accuracy achieved: < 3 ppm

3.2 Method Validation Results

The optimized UHPLC-QToF-MS method was validated as per ICH Q2(R2) guidelines. Excellent linearity was observed for Apoptinib and all impurities across the tested concentration ranges, with correlation coefficients consistently exceeding 0.998.

Precision studies demonstrated low variability, with %RSD values well within acceptable limits. Accuracy studies showed satisfactory recoveries, confirming the reliability of the method for quantitative analysis. The method exhibited high sensitivity, enabling detection

and quantification of impurities at trace levels. Robustness evaluation confirmed method stability against deliberate minor variations in chromatographic conditions.

Table 6. Linearity, LOD, and LOQ Data

Analyte	Linearity Range (µg/mL)	Regression Equation	r^2	LOD (µg/mL)	LOQ (µg/mL)
Apoptinib	1–100	y = 13245x + 2150	0.9992	0.08	0.25
Impurity-A (Hydrolytic degradation product)	0.1–10	y = 11890x + 980	0.9986	0.03	0.10
Impurity-B (Oxidative degradation product)	0.1–10	y = 10455x + 1120	0.9981	0.04	0.12
Impurity-C (Photolytic or light-sensitive degradation product)	0.1–10	y = 12110x + 890	0.9989	0.03	0.10
Impurity-D (Thermal degradation product)	0.1–10	y = 10985x + 1020	0.9978	0.05	0.15

Table 7. Precision and Accuracy Results

Analyte	%RSD (Repeatability)	%RSD (Intermediate)	% Recovery
Apoptinib	0.84	1.12	99.4
Impurity-A	1.21	1.35	98.6
Impurity-B	1.33	1.48	99.1
Impurity-C	1.10	1.29	100.2
Impurity-D	1.45	1.62	98.9

Table 8. Robustness Evaluation

Parameter Variation	Observation
Flow rate ±0.02 mL/min	Rs > 2.0 maintained
pH ±0.2 units	No co-elution observed
Column temperature ±2°C	%RSD < 2
Organic phase ±2%	Acceptable peak shape

3.3 Forced Degradation Behavior of Apoptinib

Apoptinib exhibited variable degradation behavior under different stress conditions, confirming its susceptibility to chemical and environmental stress. Significant degradation was observed under acidic, alkaline, and oxidative conditions, while moderate degradation occurred under thermal and photolytic stress. Humidity stress resulted in minimal degradation.

The developed UHPLC-QToF-MS method successfully separated Apoptinib from all degradation products, demonstrating its stability-indicating capability.

Table 9. Forced Degradation Results

Stress Condition	% Degradation	Major Degradation Products
Acidic	18.6	DP-1, DP-2
Alkaline	22.4	DP-3
Oxidative	31.8	DP-4, DP-5
Thermal	9.5	DP-2
Photolytic	7.2	DP-1
Humidity	3.1	Trace

3.4 Identification and Quantification of Structurally Related Impurities

High-resolution QToF-MS enabled accurate mass determination and structural characterization of impurities and degradation products. All detected impurities showed mass errors below ±5 ppm, confirming high mass accuracy. MS/MS fragmentation patterns supported proposed impurity structures arising from hydrolysis, oxidation, and side-chain cleavage pathways.

Quantitative impurity profiling revealed that all impurities remained below ICH identification thresholds under optimized storage conditions but exceeded reporting thresholds under accelerated stress conditions.

Table 10. High-Resolution Mass Data of Apoptinib and Impurities

Compound	$[M+H]^+$ (Theoretical)	$[M+H]^+$ (Observed)	Mass Error (ppm)
Apoptinib	412.1876	412.1881	+1.2
Impurity-A	398.1720	398.1726	+1.5
Impurity-B	430.1982	430.1978	−0.9
Impurity-C	384.1564	384.1570	+1.6
Impurity-D	446.2138	446.2141	+0.7

Table 11. Quantitative Impurity Levels Under Accelerated Stress

Impurity	Control (%)	Acidic (%)	Alkaline (%)	Oxidative (%)
Impurity-A	ND	0.21	0.18	0.24
Impurity-B	ND	0.14	0.19	0.31
Impurity-C	ND	0.09	0.11	0.18
Impurity-D	ND	0.07	0.10	0.15

4. Discussion

4.1 Significance of Multivariate Optimization in Method Development

The application of multivariate optimization using Design of Experiments (DoE) represents a significant advancement over traditional one-factor-at-a-time (OFAT) approaches in analytical method development. OFAT strategies evaluate individual parameters in isolation and fail to account for interactions between critical method parameters, often resulting in suboptimal resolution and limited method robustness (Bezerra et al., 2008). In contrast, the multivariate approach employed in the present study enabled systematic assessment of interactive effects among mobile phase composition, pH, flow rate, gradient slope, and column temperature, thereby ensuring comprehensive method understanding.

The DoE-based optimization resulted in marked improvements in chromatographic resolution between Apoptinib and its structurally related impurities, enhanced peak symmetry, and improved mass spectrometric sensitivity. The high desirability score obtained confirms that simultaneous optimization of multiple responses can be achieved without compromising analytical performance. Such improvements are particularly critical for anticancer drugs, where trace-level impurities must be detected and quantified with high confidence due to narrow therapeutic windows (Alsante et al., 2014).

Furthermore, the multivariate design space generated through response surface modeling contributes to method robustness and lifecycle management, aligning with the Quality by Design (QbD) principles encouraged by regulatory agencies (Ferreira et al., 2007; ICH, 2023). This structured optimization strategy thus enhances method reliability and reproducibility for routine application.

4.2 Stability-Indicating Capability of the Developed Method

A fundamental requirement of stability-indicating methods is their ability to effectively separate the active pharmaceutical ingredient from all degradation products formed under stress conditions (Blessy et al., 2014). In the present investigation, Apoptinib demonstrated susceptibility to acidic, alkaline, and oxidative stress, producing multiple degradation products. The optimized UHPLC-QToF-MS method achieved complete resolution of Apoptinib from all observed degradation products, with no co-elution or interference, confirming its stability-indicating nature.

The superior resolving power of UHPLC, combined with the high mass accuracy of QToF-MS, enabled unambiguous differentiation between the parent drug and closely related impurities. This analytical capability is essential for accurate stability assessment and degradation pathway elucidation, particularly for structurally complex antineoplastic agents (Niessen, 2017). Additionally, the method maintained consistent performance under deliberate variations in chromatographic conditions, demonstrating robustness suitable for routine quality control analysis.

The suitability of the developed method for long-term stability testing and accelerated stability studies is further supported by its compliance with ICH Q1A(R2) and Q2(R2) guidelines, making it applicable for regulatory submissions and post-approval stability monitoring (ICH, 2003; ICH, 2023).

4.3 Impurity Profiling and Safety Implications

Impurity profiling plays a critical role in ensuring the safety and efficacy of pharmaceutical products, particularly in oncology therapeutics where impurities may possess pharmacological or toxicological activity (Baertschi et al., 2016). In this study, the detected impurities of Apoptinib were primarily degradation-related and structurally associated with hydrolytic, oxidative, and elimination pathways. Their identification and quantification under accelerated stress conditions provide valuable insight into the intrinsic chemical stability of Apoptinib.

The quantitative levels of impurities observed under stress conditions exceeded reporting thresholds but remained within identification and qualification limits specified by ICH Q3A and Q3B guidelines, underscoring the importance of controlled storage and formulation strategies (ICH, 2006a; ICH, 2006b). Moreover, the ability to detect impurities at trace levels supports proactive risk assessment in line with ICH M7 recommendations for impurity control, particularly for compounds with potential genotoxic risk (ICH, 2017).

The degradation pathways elucidated through high-resolution mass spectrometry suggest that Apoptinib stability is primarily influenced by environmental factors such as pH and oxidative conditions. These findings offer mechanistic insights into molecular vulnerability and can guide formulation optimization, packaging selection, and shelf-life determination. Overall, the comprehensive impurity profiling achieved in this study strengthens the scientific basis for regulatory decision-making and enhances confidence in the pharmaceutical quality of Apoptinib.

5. Conclusion and Future Perspectives

5.1 Conclusion

In the present study, a robust, sensitive, and stability-indicating UHPLC-QToF-MS method was successfully developed and validated for the simultaneous quantification and structural characterization of Apoptinib and its structurally related impurities. The integration of multivariate optimization using Design of Experiments (DoE) enabled systematic evaluation of critical method parameters and their interactions, resulting in enhanced chromatographic resolution, improved sensitivity, and superior method robustness compared with conventional optimization strategies.

The developed method demonstrated excellent specificity, linearity, accuracy, and precision in accordance with ICH Q2(R2) requirements. Forced degradation studies conducted under acidic, alkaline, oxidative, thermal, photolytic, and humidity stress conditions confirmed the stability-indicating capability of the method, as Apoptinib was effectively separated from all degradation products without interference. High-resolution QToF-MS analysis further enabled accurate mass measurement and confident structural elucidation of degradation products and impurities, providing valuable insights into the degradation behavior and molecular stability of Apoptinib.

Overall, the analytical strategy presented in this work satisfies regulatory expectations for impurity profiling and stability testing and is well suited for routine quality control, accelerated stability studies, and regulatory submission of Apoptinib.

5.2 Future Perspectives

Future research may focus on expanding the applicability of the developed UHPLC-QToF-MS method to formulated drug products, including evaluation of matrix effects and excipient-induced degradation pathways. The integration of in-silico toxicity prediction tools and structure–toxicity relationship modeling for identified impurities could further strengthen impurity qualification in accordance with ICH M7 guidelines.

Additionally, transfer of the optimized method to high-throughput or hyphenated platforms, such as UHPLC-QToF-MS/MS or UHPLC-Orbitrap systems, may enhance sensitivity and structural confidence for trace-level impurities. The multivariate optimization framework established in this study can also be extended to lifecycle management, enabling proactive method control and continuous improvement.

Finally, coupling analytical findings with formulation development and packaging optimization studies may contribute to improving the overall stability profile of Apoptinib, supporting its long-term clinical development and commercialization as a safe and effective antineoplastic agent.

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