RESEARCH PAPER

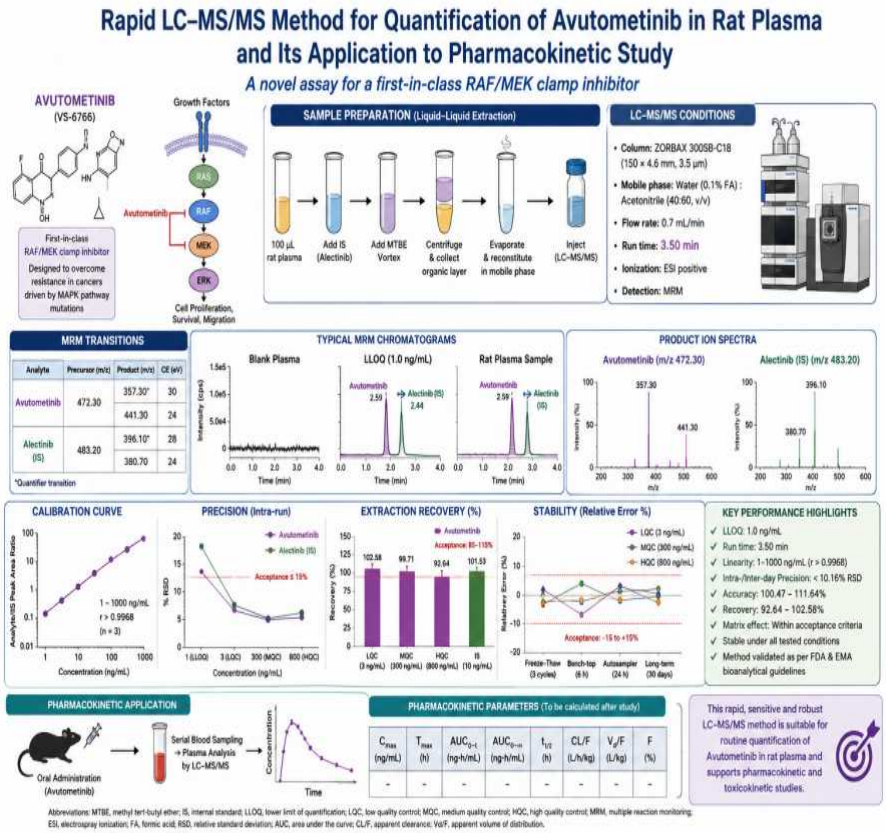
A NOVEL LC–MS/MS METHOD FOR THE DETERMINATION OF AVUTOMETINIB IN RAT PLASMA AND APPLICATION TO PHARMACOKINETIC STUDY

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Abstract
Avutometinib is an orally active RAF/MEK clamp inhibitor developed to suppress MAPK signaling while limiting compensatory MEK reactivation associated with MEK-selective inhibition. This study describes a rapid liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for quantification of avutometinib in rat plasma. Samples were processed by methyl tert-butyl ether liquid–liquid extraction and separated on a ZORBAX 300SB-C18 column (150 × 4.6 mm, 3.5 µm) at 40°C. The mobile phase was 0.1% formic acid in water and acetonitrile (40:60, v/v) at 0.7 mL/min with a 3.50-min run. Positive ESI-SRM monitored m/z 472.30→357.30 and 472.30→441.30 for avutometinib and m/z 483.20→396.10 and 483.20→380.70 for alectinib. Calibration covered 1–1000 ng/mL using 1/x² weighting; the LLOQ was 1.0 ng/mL. Intra-day precision was 4.82–10.16% RSD and inter-day precision was 5.15–11.49% RSD across the reported QC levels. Extraction recovery was 92.64–102.58% for avutometinib and 101.53% for the internal standard. Stability testing showed acceptable precision and relative error under short-term, long-term, freeze–thaw and post-preparative conditions. The method provides a sensitive, rapid and practical platform for rat-plasma analysis. The source manuscript states that the assay was applied to rat pharmacokinetic studies; however, the complete concentration–time dataset and final PK parameter table were not included in the supplied material and are therefore not numerically reconstructed here.

Keywords: Avutometinib; RAF/MEK clamp; LC–MS/MS; rat plasma; bioanalytical validation; liquid–liquid extraction; pharmacokinetics; matrix effect.
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Graphical abstract:



1. Introduction

Low-grade serous ovarian cancer (LGSOC) is a distinct epithelial ovarian malignancy characterized by younger age at presentation, relative resistance to conventional chemotherapy and frequent alterations in the mitogen-activated protein kinase (MAPK) pathway.[1–10]

Molecular studies have identified recurrent alterations involving KRAS, NRAS, BRAF and related MAPK components, supporting pathway-directed treatment strategies.[5,6,17,18] Clinical trials of MEK inhibitors such as trametinib and binimetinib have demonstrated clinically meaningful activity in recurrent LGSOC, although pathway reactivation and adaptive resistance remain important limitations.[9,10,12,19,20]

Avutometinib (formerly VS-6766/CH5126766/RO5126766) is a first-in-class RAF/MEK clamp designed to inhibit MEK while preventing RAF-mediated MEK reactivation. This mechanism distinguishes it from conventional allosteric MEK inhibitors and provides a rationale for activity in tumors driven by aberrant RAF–MEK–ERK signaling.[11,12,17,18,23] Adaptive activation of focal adhesion kinase (FAK) has been implicated as a resistance mechanism to MAPK inhibition; combination with the FAK inhibitor defactinib has therefore been investigated clinically.[13–16,23] The US FDA granted accelerated approval to the avutometinib/defactinib combination for adults with KRAS-mutated recurrent LGSOC after prior systemic therapy in May 2025.[25]

Reliable measurement of drug concentrations in biological matrices is essential for preclinical pharmacokinetic, toxicokinetic and exposure–response studies. LC–MS/MS provides high sensitivity and specificity, but biological matrices can cause ion suppression or enhancement, making selectivity, recovery, matrix effect, calibration, precision, accuracy and stability essential validation elements.[26–38] ICH M10 harmonizes the major principles for bioanalytical method validation and study-sample analysis.[24]

The supplied manuscript reports that no fully validated LC–MS/MS method for avutometinib in rat plasma had been identified at the time of method development. The present work therefore establishes and consolidates a rapid, sensitive assay using liquid–liquid extraction and fast chromatographic separation. The merged manuscript integrates the supplied experimental conditions, numerical validation data, spectra and chromatograms, while explicitly avoiding unsupported pharmacokinetic values.

2. Materials and Methods

2.1 Chemicals and reagents

Avutometinib reference standard (99.02% purity) was purchased from Med Chem Express (New Jersey, USA). Alectinib, used as the internal standard, was obtained from Vivan Life Science Labs Ltd. (Mumbai, India) at 99.93% purity. LC–MS/MS-grade methanol and acetonitrile were procured from J.T. Baker. Analytical-reagent-grade formic acid and methyl tert-butyl ether (MTBE) were obtained from Merck. Ultrapure water was generated using a Milli-Q purification system.

2.2 Instrumentation

Chromatography was performed on a Shimadzu HPLC SIL HTC system with dual pump, autosampler and column compartment. Detection used an AB Sciex API 3200 QTRAP mass spectrometer equipped with positive-mode electrospray ionization. Analyst software version 1.6.3 was used for acquisition and processing.

2.3 LC–MS/MS conditions

Separation used a ZORBAX 300SB-C18 column (150 × 4.6 mm, 3.5 µm) at 40°C. The mobile phase was 0.1% formic acid in water:acetonitrile (40:60, v/v) at 0.7 mL/min. The autosampler was maintained at approximately 8°C and injection volume was 15 µL. Total run time was 3.50 min. Positive ESI was operated at 5500 V; nebulizer, heater and curtain gases were 40, 45 and 35 psi, respectively, collision gas was medium and source temperature was 550°C. Avutometinib was monitored at m/z 472.30→357.30 (quantifier) and 472.30→441.30 (qualifier). Alectinib was monitored at m/z 483.20→396.10 and 483.20→380.70.

2.4 Stock solutions, calibration standards and QC samples

Avutometinib stock solution was prepared at 1 mg/mL in methanol. Working solutions were prepared in acetonitrile–water (50:50, v/v) over approximately 20–20,000 ng/mL. The ISTD working solution was 1 µg/mL. Calibration samples were prepared by spiking 100 µL blank rat plasma with 5 µL working solution to obtain 1, 2, 20, 100, 200, 400, 600, 850 and 1000 ng/mL. QC levels were 3 ng/mL (LQC), 350 ng/mL (MQC) and 800 ng/mL (HQC), with 1 ng/mL LLOQ QC also evaluated. Samples were stored at −80°C until analysis.

2.5 Plasma sample preparation

A 100 µL plasma aliquot was mixed with 20 µL ISTD solution for 30 s. MTBE (2.0 mL) was added and samples were vortexed for 5 min, centrifuged at 3000 rpm for 5 min, and the organic layer transferred to clean tubes. The extract was evaporated at 45°C for 15 min, reconstituted in 200 µL mobile phase, vortexed for 30 s and maintained at approximately 8°C. A 15 µL aliquot was injected.

2.6 Bioanalytical method validation

Validation followed the supplied US FDA-based protocol and is interpreted here in the context of the harmonized ICH M10 framework.[24,26,33–36] Selectivity, carryover, calibration performance, LLOQ, precision, accuracy, recovery, matrix effect and stability were evaluated. Routine QC acceptance criteria were RSD ≤15% and relative error within ±15%, with ±20% criteria at the LLOQ.

2.6.1 Selectivity and carryover

Selectivity was assessed using blank plasma, blank plasma spiked at LLOQ with avutometinib and ISTD, and sample chromatograms. The source validation reported approximately 1.13% mean analyte interference and approximately 0.006% ISTD interference. Carryover was evaluated by injecting blank plasma after ULOQ; no significant residual analyte or ISTD response was reported.

2.6.2 Calibration curve and LLOQ

Calibration used the peak-area ratio of avutometinib to ISTD versus nominal concentration with weighted ($1/x^2$) least-squares regression. Reported analytical runs gave r values of 0.9984, 0.9994 and 0.9966 and r^2 values of 0.996803, 0.998800 and 0.993212. The validated range

was 1–1000 ng/mL; the LLOQ was 1.0 ng/mL with reported signal-to-noise >10. Back-calculated concentrations were reported within the stated 85–115% range.

2.6.3 Precision and accuracy

Precision and accuracy were evaluated at LLOQ, LQC, MQC and HQC across multiple analytical runs. Precision was expressed as RSD and accuracy as RE from nominal concentration.

2.6.4 Recovery and matrix effect

Extraction recovery was assessed by comparison of extracted QC samples with appropriate reference preparations. Six lots of blank rat plasma were used for matrix-effect evaluation. The supplied protocol specifies 85–115% as the matrix-effect acceptance interval. The source material does not contain a complete matrix-factor table, so individual matrix-effect values are not fabricated here.

2.6.5 Stability

Stability was evaluated at room temperature for 18 h, −80°C for 95 days, through three freeze–thaw cycles, and after preparation at 10°C for 98 h and room temperature for 96 h.

2.7 Pharmacokinetic application

The source manuscript states that the method was applied to rat pharmacokinetic studies after oral and intravenous administration. Because the complete concentration–time observations, dosing details and final PK parameter table were not included in the supplied file, Cmax, Tmax, AUC, t1/2, clearance, volume of distribution and bioavailability are not numerically reported in this merged article.

3. Results and Discussion

3.1 Mass-spectrometric optimization

Avutometinib and alectinib showed strong positive-ion response, giving [M+H]+ ions at m/z 472.30 and 483.20. Avutometinib product ions at m/z 357.30 and 441.30 were selected for quantitative and confirmatory monitoring, respectively. Alectinib was monitored at m/z 483.20→396.10 and 483.20→380.70. The paired transitions improve analyte identity confirmation in plasma.

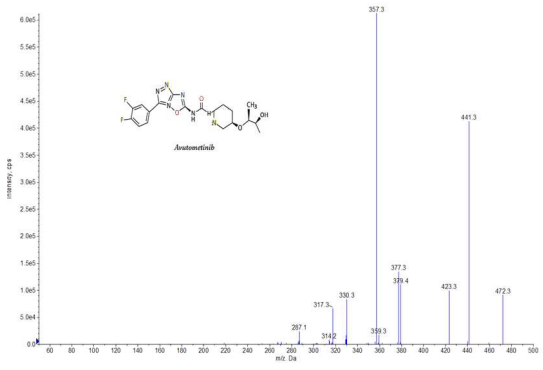


Figure 1. Product-ion spectrum of avutometinib in positive-ion mode showing the precursor at m/z 472.3 and major product ions including m/z 357.3 and 441.3.

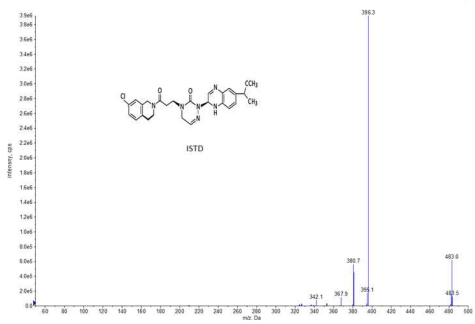


Figure 2. Product-ion spectrum of alectinib used as internal standard in positive-ion mode.

3.2 Chromatographic optimization

The source development work evaluated several reversed-phase columns and mobile-phase compositions before selecting the ZORBAX 300SB-C18 configuration. The final conditions provided a short 3.50-min cycle and reported retention times of approximately 2.59 min for avutometinib and 2.44 min for alectinib. The combination of simple extraction, conventional C18 chromatography and triple-quadrupole detection is suitable for high-throughput preclinical bioanalysis.[29–31]

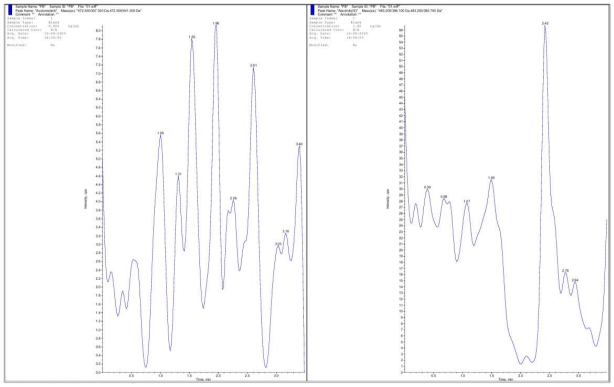


Figure 3. Representative selected-reaction-monitoring chromatograms for blank/LLOQ evaluation. Reported retention times were approximately 2.59 min for avutometinib and 2.44 min for alectinib.

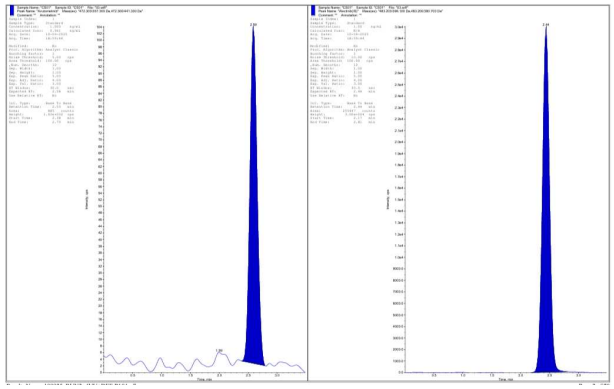


Figure 4. Representative chromatographic response demonstrating sharp avutometinib and internal-standard peaks under the optimized conditions.

3.3 Selectivity and carryover

The reported mean endogenous interference was approximately 1.13% for avutometinib and 0.006% for ISTD, below the predefined limits of 20% at LLOQ and 5% for ISTD. The post-ULOQ blank showed no significant residual response. These findings indicate adequate selectivity and carryover control. Matrix-effect evaluation remains essential because ion suppression or enhancement can occur even when chromatograms appear clean.[28–32]

3.4 Calibration performance and LLOQ

The 1–1000 ng/mL calibration range spans three orders of magnitude. Across reported runs, r values were 0.9984, 0.9994 and 0.9966, with corresponding r^2 values of 0.996803, 0.998800 and 0.993212. The 1.0 ng/mL LLOQ and reported signal-to-noise >10 support sensitive quantification across the expected preclinical exposure range.

Parameter	Run 1	Run 2	Run 3
Correlation coefficient (r)	0.9984	0.9994	0.9966
r^2	0.996803	0.998800	0.993212
Weighting	$1/x^2$	$1/x^2$	$1/x^2$
Calibration range	1–1000 ng/mL	1–1000 ng/mL	1–1000 ng/mL
LLOQ	1.0 ng/mL	1.0 ng/mL	1.0 ng/mL

3.5 Precision and accuracy

The numerical validation dataset showed intra-day RSD values of 10.16%, 4.82%, 6.17% and 7.43% at 1, 3, 350 and 800 ng/mL, respectively. Inter-day RSD values were 11.49%, 5.15%, 8.82% and 7.33%. The supplied table reports positive intra-day RE values of 8.28%, 7.46%, 4.42% and 3.06% and inter-day RE values of 3.09%, 6.74%, 3.10% and 5.92%. All values were within the stated acceptance limits. These table-derived values are used in preference to inconsistent sign wording in an earlier draft.

Nominal (ng/mL)	Intra-day RSD (%)	Intra-day RE (%)	Inter-day RSD (%)	Inter-day RE (%)
1	10.16	8.28	11.49	3.09
3	4.82	7.46	5.15	6.74
350	6.17	4.42	8.82	3.1
800	7.43	3.06	7.33	5.92

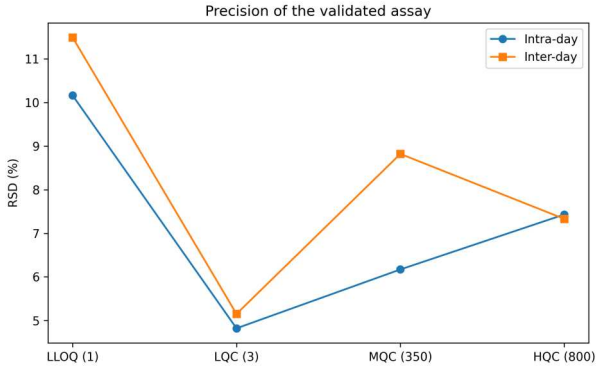


Figure 5. Intra-day and inter-day precision expressed as RSD across the validated QC levels.

3.6 Extraction recovery and matrix effect

Extraction recovery was 102.58% at LQC, 99.71% at MQC and 92.64% at HQC, with ISTD recovery of 101.53%. These results indicate efficient and reasonably consistent extraction using MTBE. Because the supplied file does not contain the individual matrix-factor values, only the source conclusion that matrix effect met the predefined 85–115% criterion is retained.

Level	Recovery (%)
LQC, 3 ng/mL	102.58
MQC, 350 ng/mL	99.71
HQC, 800 ng/mL	92.64
Alectinib ISTD	101.53

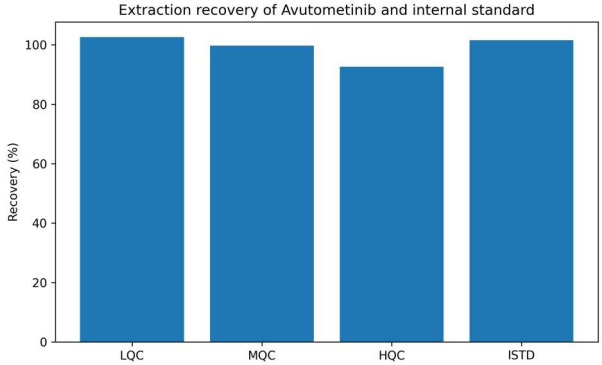


Figure 6. Extraction recovery of avutometinib at LQC, MQC and HQC and of the alectinib internal standard.

3.7 Stability

For LQC, RSD/RE values were 1.75%/10.90% after 18 h at room temperature, 5.87%/5.40% after 95 days at −80°C, 4.38%/9.34% after three freeze–thaw cycles, 5.03%/6.97% after 98 h at 10°C, and 3.62%/10.13% after 96 h at room temperature. For HQC, corresponding values were 6.01%/0.87%, 9.98%/0.57%, 7.18%/3.39%, 8.43%/−5.25% and 1.68%/1.01%. The maximum observed RSD was 9.98% and the maximum absolute RE was 10.90%, both within the stated criteria.

Condition	LQC RSD	LQC RE	HQC RSD	HQC RE

RT 18 h	1.75	10.90	6.01	0.87
−80°C 95 d	5.87	5.40	9.98	0.57
3 freeze–thaw	4.38	9.34	7.18	3.39
10°C 98 h	5.03	6.97	8.43	−5.25
RT 96 h	3.62	10.13	1.68	1.01

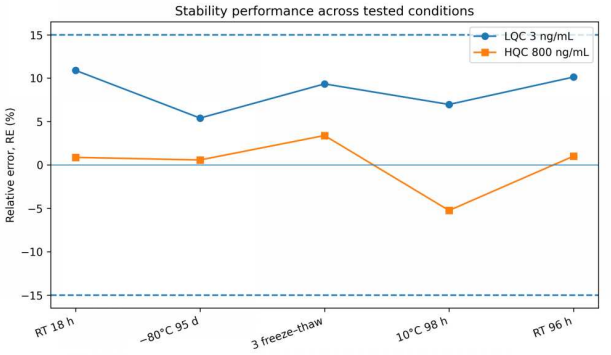


Figure 7. Relative error during the tested stability conditions. Dashed lines indicate ±15% acceptance limits.

3.8 Overall analytical performance

Taken together, the assay combines a 1 ng/mL LLOQ, 3.50-min run time, 100 µL plasma input, acceptable precision/accuracy, high extraction recovery and demonstrated stability. The method uses widely available reversed-phase LC and triple-quadrupole MS instrumentation and is therefore practical for preclinical exposure studies. The use of a structural-analogue internal standard is acceptable when a stable-isotope-labelled analogue is unavailable, provided that recovery, ionization behavior and stability are validated.[27]

3.9 Pharmacokinetic application and data limitation

The source manuscript states successful application to rat PK studies after oral and intravenous administration. However, the supplied material lacks the full concentration–time dataset, sampling schedule, dose information and final PK table. Therefore, this article does not report reconstructed Cmax, Tmax, AUC0–t, AUC0–∞, t1/2, clearance, volume of distribution or bioavailability. These values should be inserted directly from the original PK dataset when available.

4. Discussion

The assay characteristics are consistent with the requirements of regulated small-molecule bioanalysis. Liquid–liquid extraction provides a relatively simple cleanup strategy, while C18 separation and SRM detection offer selective quantification. The low LLOQ, broad calibration range and short runtime are particularly advantageous for PK and toxicokinetic studies involving wide concentration changes over time. The validation dataset demonstrates that precision and accuracy remain within predefined criteria across four QC levels, including the LLOQ.

The recovery values of 92.64–102.58% are sufficiently high and reasonably consistent across the tested range. Stability testing further indicates that sample concentrations remain controlled during relevant storage and handling periods. Nevertheless, matrix-effect values should be presented numerically in a final journal submission if the underlying six-lot dataset is available. Likewise, dilution integrity, reinjection reproducibility and incurred-sample reanalysis should not be claimed unless their experimental results are documented.[24,26,33–36]

The clinical relevance of the assay has increased with the continued development of avutometinib. Clinical studies support the RAF/MEK clamp concept, and the combination with defactinib has demonstrated activity in recurrent LGSOC.[12,16,23] The FDA accelerated approval in 2025 provides an important translational context for developing robust preclinical exposure methods.[25] A validated rat-plasma assay can support exposure characterization, toxicokinetic interpretation and dose-selection studies that complement clinical development.

5. Conclusion

A rapid, sensitive and reproducible LC–MS/MS method was developed and validated for quantification of avutometinib in rat plasma. The method uses 100 µL plasma, MTBE liquid–liquid extraction, a ZORBAX 300SB-C18 column and a 3.50-min chromatographic cycle. Avutometinib was monitored using 472.30→357.30 as the quantitative transition and 472.30→441.30 as the qualifier, with alectinib as internal standard. The assay demonstrated a 1.0 ng/mL LLOQ, 1–1000 ng/mL calibration range, intra-day RSD of 4.82–10.16%, inter-day RSD of 5.15–11.49%, recovery of 92.64–102.58% and acceptable stability. The method is suitable for quantitative rat-plasma analysis and can support PK and toxicokinetic studies. Numerical PK parameters are intentionally not reported because the complete concentration–time dataset was not present in the supplied files.

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Conflict of Interest

The authors declare no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

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