Quantitative Structure–Cytotoxicity Relationship of Piperic Acid Amides

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Abstract. Background: A total of 12 piperic acid amides, including piperine, were subjected to quantitative structure–activity relationship (QSAR) analysis, based on their cytotoxicity, tumor selectivity and anti-HIV activity, in order to find new biological activities. Materials and Methods: Cytotoxicity against four human oral squamous cell carcinoma (OSCC) cell lines and three human oral normal cells was determined by the 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. Tumor selectivity was evaluated by the ratio of the mean 50% cytotoxic concentration (CC50) against normal oral cells to that against OSCC cell lines. Anti-HIV activity was evaluated by the ratio of the CC50 to 50% HIV infection-cytoprotective concentration (EC50). Physicochemical, structural, and quantum-chemical parameters were calculated based on the conformations optimized by LowModeMD method followed by density functional theory method. Results: All compounds showed low-to-moderate tumor selectivity, but no anti-HIV activity. N-Piperyldopamine (8) which has a catechol moiety, showed the highest tumor selectivity, possibly due to its unique molecular shape and electrostatic interaction, especially its largest partial equalization of orbital electronegativities and vsurf descriptors. Conclusion: The present study suggests that molecular shape and ability for electrostatic interaction are useful parameters for estimating the tumor selectivity of piperic acid amides.

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Key Words: Piperic acid amides, QSAR analysis, cytotoxicity, tumor selectivity, anti-HIV activity.
For the cytotoxicity assay, both human normal oral cells (gingival fibroblast, HGF; periodontal ligament fibroblast, HPLF; pulp cells, HPC) and human oral squamous cell carcinoma (OSCC) cell lines (Ca9-22, HSC-2, HSC-3, HSC-4) were used as target cells. The antitumor potential was evaluated by the tumor-selectivity index (TS), calculated by dividing the mean 50% cytotoxic concentration (CC50) against normal oral cells by that against OSCC cell lines. We have recently reported that among 24 plant extracts, leaves of *Camptotheca acuminate*, a well-known source of camptothecin, had the highest TS value (88.3) among 24 plant extracts, suggesting that the TS value determined by this method seems to reflect the antitumor potential of each plant extract, although these oral normal and OSCC cell lines of oral origin are classified as different types of cells (mesenchymal or epithelial) (19).

For the anti-HIV assay, mock- and HIV-infected human T-cell lymphotropic virus-I (HTLV-I)-carrying human T-cell line MT4 was used. The selectivity index (SI) was calculated by dividing the CC50 by the 50% HIV infection-cytoprotective concentration (EC50).

**Materials and Methods**

*Materials.* The following chemicals and reagents were obtained from the indicated companies: Dulbecco’s modified Eagle’s medium (DMEM), from GIBCO BRL, Grand Island, NY, USA; fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), azidothymidine and 2',3'-dideoxycytidine from Sigma-Aldrich Inc., St. Louis, MO, USA; piperine, dimethyl sulfoxide (DMSO), dextran sulfate (molecular mass, 5 kDa) from Wako Pure Chem. Ind., Osaka, Japan; 5-fluorouracil (5-FU) from Kyowa, Tokyo, Japan; curdlan sulfate (molecular mass, 79 kDa) from Ajinomoto Co. Ltd., Tokyo, Japan. Culture plastic dishes and
plates (96-well) were purchased from Becton Dickinson Labware (Franklin Lakes, NJ, USA).

Synthesis of test compounds. N-Piperoyl-ethanolamine (2), N-piperoylpiperazine (3), N-piperoylcadaverine (4), N-piperoylphe- 
nethyamine (5), N-piperoyl-3-phenylpropylamine (6), N- 
piperoyltyramine (7), N-piperoyldopamine (8), N-piperoylvanil- 
lamine (9), N-piperoylerosamine (10), N-piperoylhistamine (11) and 
N-piperoyl-2-(2-pyridinyl)ethylamine (12) (Figure 1) were 
synthesized by coupling of piperic acid with the appropriate amine 
by means of a modified procedure described elsewhere (20). To 
a mixture of piperic acid (1.0 mmol) in CH$_2$Cl$_2$ (5 ml) was added 
oxalyl chloride (10 mmol), and the mixture was stirred at room 
temperature for 3 h. The solvent and excess oxalyl chloride were 
then evaporated under reduced pressure. The crude acid chloride 
generated was dissolved in CH$_2$Cl$_2$ or dimethylformamide (DMF) 
(2 ml), and was added dropwise to a mixture of the appropriate amine 
or its hydrochloride salt (1.2 mmol) and Et$_3$N (8 mmol) in 
CH$_2$Cl$_2$ or DMF (5 ml) under ice-cooling. The reaction mixture 
was stirred for 5 h at room temperature. Ice-water was added to 
the mixture and the whole was extracted with CHCl$_3$. The organic 
layer was dried over Na$_2$SO$_4$ and the solvent was evaporated under 
reduced pressure. The residue was then purified by silica gel 
column chromatography to give the corresponding piperic acid 
amide. All the conjugates were characterized by $^1$H nuclear 
resonance (NMR) and mass spectrometry (MS) data. All 
compounds were dissolved in DMSO at 40 mM and stored at 
$-20^\circ$C before use.

Cell culture. HGF, HPLF and HPC cells, established from the first 
premolar tooth extracted from the lower jaw of a 12-year-old girl 
(21), and OSCC cell lines (Ca9-22, HSC-2, HSC-3, HSC-4), purchased from Riken Cell Bank, Tsukuba, Japan were cultured at 
37°C in DMEM supplemented with 10% heat-inactivated FBS, 100 
units/ml, penicillin G and 100 μg/ml streptomycin sulfate under a 
humidified atmosphere with 5% CO$_2$. Cells were then harvested by 
treatment with 0.25% trypsin-0.025% EDTA-2Na in phosphate-
buffered saline without calcium and magnesium [PSB(−)] and either 
subcultured or used for experiments.

Assay for cytotoxic activity. Cells were inoculated at 2.5x10$^3$ cells/0.1 ml in a 96-microwell plate (Becton Dickinson Labware). 
After 48 h, the medium was removed by suction with aspirator, and 
replaced with 0.1 ml of fresh medium containing different 
fractions of single test compounds. Control cells were treated 
with the same amounts of DMSO present in each diluent solution. 
Cells were incubated for 48 h, and the relative viable cell number 
was then determined by MTT method. In brief, the treated cells 
were incubated for another 3 h in fresh culture medium containing 
0.2 mg/ml MTT. Cells were then lysed with 0.1 ml of DMSO, and 
the absorbance at 540 nm of the cell lysate was determined using a 
microplate reader (Biochromatic Labsystem, Helsinki, Finland). 
The CC$_{50}$ was determined from the dose—response curve and the mean 
value of CC$_{50}$ for each cell type was calculated from three 
independent experiments.

Calculation of TS. The TS was calculated by the following equation: 
TS=mean CC$_{50}$ against normal cells/mean CC$_{50}$ against tumor cells. 
Since Ca9-22 cells were derived from gingival tissue (22), the 
relative sensitivity of Ca9-22 and HGF was also compared.

Assay for HIV activity. HTLV-I-carrying human T-cell line MT4 
cells (supplied by Dr. Naoki Yamamoto), highly sensitive to Human 
Immunodeficiency Virus-1 (HIV-1), were infected with HIV-1_LIB 
at a multiplicity of infection (m.o.i.) of 0.01. HIV- and mock-infected 
(control) MT-4 cells were incubated for five days with different 
fractions of test compounds and the relative viable cell number 
was determined by MTT assay. The CC$_{50}$ and EC$_{50}$ were 
determined from the dose—response curve for mock-infected and 
HIV-infected cells, respectively (23). All data represent the mean 
values of triplicate measurements. The anti-HIV activity was 
evaluated by SI (CC$_{50}$/EC$_{50}$).

Estimation of CC$_{50}$ values. Original data contain the sign of 
inequality such as“>”. For the convenience of analysis, these values 
were changed into forms suitable for arithmetic calculation. Since 
“>400” is equal to “from 400 to ∞”, we calculated the harmonic 
mean as follows: 1/average(1/400,1/∞)=800. Since the CC$_{50}$ values 
had a distribution pattern close to a logarithmic normal distribution, 
we used the pCC$_{50}$ (i.e., the $-\log$ CC$_{50}$) for the comparison of the 
cytotoxicity between the compounds. The mean pCC$_{50}$ values for 
normal cells and tumor cell lines were defined as N and T, 
respectively (24).

Calculation of the representative value for tumor selectivity. Tumor 
selectivity is defined by the balance between pCC$_{50}$ values for 
normal (N) and tumor (T) cells. The difference (T−N) was used as 
a tumor-selectivity index only for the following QASR analyses.

Calculation of chemical descriptors. Each chemical structure was 
optimized by the LowModeMD method (25), a suitable search 
method for minimum energy conformers of flexible molecules, with 
Merck Molecular Force Field (MMFF94x) in Molecular Operating 
Environment (MOE) 2013.08 (Chemical Computing Group Inc., 
Quebec, Canada). Each structure was refined with density functional 
theory (DFT-B3LYP/6-31G**) by using Spartan10 for Windows 
(Wavefunction, Inc., Irvine, CA, USA) (26). During each step of the 
calculation, quantum chemical, molecular shape, and molecular 
property parameters including the partial equalization of orbital 
electronatgbivties (PEOE) and vsurf descriptors, were obtained. The 
parameters used were: a$_{hyd}$ (number of hydrophobic atoms), a$_{nO}$ 
(number of oxygen atoms), logP(o/w) (log of the octanol/water 
partition coefficient), logS (log of the aqueous solubility), 
PEOE_VSA_FNEG (fractional negative van der Waals surface area), 
PEOE_VSA_FPOS (fractional positive van der Waals surface area), 
PEOE_VSA_NEG (total negative van der Waals surface area), 
PEOE_VSA_PNEG (total negative polar van der Waals surface area), 
PEOE_VSA_POL (total polar van der Waals surface area), 
PEOE_VSA_PPOS (total positive polar van der Waals surface area), 
PEOE_VSA+4 (sum of vi where qi is in the range 0.20-0.25; vi and 
qi denote the van der Waals surface area and the partial charge of 
a atom i, respectively), vsurf_EWmin1 (lowest hydrophilic energy 1), 
vsurf_HB6 (H-bond donor capacity 6), vsurf_HB7 (H-bond donor 
capacity 7), vsurf_IW7 (hydrophilic interaction-energy moment 7), 
vsurf_IW8 (hydrophilic interaction-energy moment 8), vsurf_W7 
(hydrophilic volume 7).

Statistical analysis. The relation among cytotoxicity, tumor-
specificity and chemical descriptors was investigated using simple 
regression analyses by JMP Pro version 10.0.2 (SAS Institute Inc., 
Cary, NC, USA). The significance level was set at p<0.05.
Results

Cytotoxicity. Compared to the positive control, 5-FU, piperine exhibited minor tumor specificity (Table I). Among 11 other analogs, compound [8] exhibited the highest tumor specificity, whereas other compounds [2-7, 9-12] exhibited much lower tumor specificity (Table I).

Anti-HIV activity. In contrast to the higher anti-HIV activity of positive controls (dextran sulfate, curdlan sulfate, azidothymidine, 2',3'-dideoxycytidine) (SI=1789-15882), none of the piperic acid amides [1-12] were able to protect cells from cytopathic effect of HIV infection (SI<1) (Table II). Based on these data, the subsequent QSAR analysis was focused on the cytotoxicity of piperic acid amides.

Computational analysis. Cytotoxicity of piperic acid amides against tumor cells (defined by T) correlated with the partial equalization of orbital electronegativity in total negative van der Waals surface area ($r^2=0.751$, $p<0.0005$), fractional positive van der Waals surface area ($r^2=0.701$, $p<0.0001$), fractional negative van der Waals surface area ($r^2=0.701$, $p<0.0001$), log of the octanol/water partition coefficient ($r^2=0.492$, $p<0.05$), number of hydrophobic atoms ($r^2=0.473$, $p<0.05$) and log of the aqueous solubility ($r^2=0.432$, $p<0.05$) (Figure 2A).

On the other hand, cytotoxicity of piperic acid amides against normal cells (defined by N) was correlated with hydrophilic interaction-energy moment 7 ($r^2=0.530$, $p<0.01$), lowest hydrophilic energy 1 ($r^2=0.491$, $p<0.05$), H-bond donor capacity 7 ($r^2=0.484$, $p<0.05$), hydrophilic volume 7 ($r^2=0.484$, $p<0.05$), H-bond donor capacity 6 ($r^2=0.476$, $p<0.05$) (Figure 2B).

Table I. Cytotoxic activity of twelve piperic acid amides. Each value represents the mean ± S.D. of triplicate assays.

<table>
<thead>
<tr>
<th>Piperic acid amide</th>
<th>Human oral squamous cell carcinoma cell line</th>
<th>Human normal oral cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ca9-22 HSC-2 HSC-3 HSC-4 mean±S.D.</td>
<td>HGF HPLF HPC mean±S.D.</td>
</tr>
<tr>
<td>Piperic acid amide</td>
<td>(A) (B) (C) (D/B) (C/A)</td>
<td>(E) (F) (G) (H/G) (I/J)</td>
</tr>
<tr>
<td>1</td>
<td>128±14 512±38 583±202 600±24 456±222</td>
<td>473±22 513±13 501±39 496±21 1.1 3.6</td>
</tr>
<tr>
<td>2</td>
<td>239±50 335±54 487±57 450±72 378±113</td>
<td>539±19 510±70 518±19 522±15 1.4 2.3</td>
</tr>
<tr>
<td>3</td>
<td>103±15 114±14 134±14 122±11 118±13</td>
<td>81±22 127±15 137±13 115±30 1.0 0.8</td>
</tr>
<tr>
<td>4</td>
<td>107±76 118±51 170±31 152±18 137±29</td>
<td>122±8.0 137±7.0 131±47 130±7.5 1.0 1.1</td>
</tr>
<tr>
<td>5</td>
<td>7.4±0.8 13±2.1 73±23 199±174 73±89</td>
<td>21±4.0 68±4.5 82±31 57±32 0.8 2.8</td>
</tr>
<tr>
<td>6</td>
<td>13±4.6 18±7.0 81±2.1 208±88 80±91</td>
<td>16±3.8 41±2.6 19±1.5 25±14 0.3 1.3</td>
</tr>
<tr>
<td>7</td>
<td>11±0.1 16±6.4 18±4.2 14±2.5 15±3.0</td>
<td>13±0.58 23±3.2 20±1.0 19±5.1 1.3 1.2</td>
</tr>
<tr>
<td>8</td>
<td>38±8.5 51±13 131±60 80±17 75±41</td>
<td>&gt;800 &gt;800 &gt;800 &gt;800 &gt;10.7 &gt;21.1</td>
</tr>
<tr>
<td>9</td>
<td>79±11 447±331 97±3.2 &gt;800 &gt;356</td>
<td>535±8.7 535±13 573±26 548±22 &lt;1.5 6.8</td>
</tr>
<tr>
<td>10</td>
<td>33±12 15±38 7.2 58±15 45±12</td>
<td>41±1.7 46±2.3 75±12 54±18 1.2 1.2</td>
</tr>
<tr>
<td>11</td>
<td>455±123 696±105 &gt;800 500±53 &gt;613</td>
<td>658±36 617±33 680±41 652±32 &lt;1.1 1.4</td>
</tr>
<tr>
<td>12</td>
<td>183±9.0 262±14 268±6.5 250±3.5 241±39</td>
<td>343±104 497±5.5 467±50 436±82 1.8 1.9</td>
</tr>
<tr>
<td>5-FU</td>
<td>88±11 24±7.8 38±7.6 28±4.9 45±30</td>
<td>&gt;1000 &gt;1000 &gt;1000 &gt;1000 &gt;22.2 &gt;11.4</td>
</tr>
</tbody>
</table>

HGF: Human gingival fibroblast; HPC, human pulp cells; HPLF, human periodontal ligament fibroblast; Ca9-22, HSC-2, HSC-3, HSC-4: human oral squamous cell carcinoma cell lines; TS: tumor-selectivity index; CC₅₀: 50% cytotoxic concentration; 5-FU: 5-fluorouracil.

Table II. Anti-HIV activity of piperic acid amides and chemotherapeutic agents. Each value represents the mean of triplicate determinations.

<table>
<thead>
<tr>
<th>Piperic acid amides</th>
<th>CC₅₀ (μM)</th>
<th>EC₅₀ (μM)</th>
<th>SI</th>
</tr>
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<tr>
<td>1</td>
<td>324</td>
<td>&gt;800</td>
<td>&lt;1</td>
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<td>2</td>
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<td>3</td>
<td>112</td>
<td>&gt;800</td>
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<td>4</td>
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<td>&gt;800</td>
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<td>5</td>
<td>36</td>
<td>&gt;800</td>
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<td>8</td>
<td>46</td>
<td>&gt;800</td>
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<td>9</td>
<td>688</td>
<td>&gt;800</td>
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<td>10</td>
<td>279</td>
<td>&gt;800</td>
<td>&lt;1</td>
</tr>
<tr>
<td>11</td>
<td>32</td>
<td>&gt;800</td>
<td>&lt;1</td>
</tr>
<tr>
<td>12</td>
<td>175</td>
<td>&gt;800</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

Positive controls

Dextran sulfate (μg/ml) | 621 | 0.05 | 12363 |
Cardiol sulfate (μg/ml) | >1000 | 0.18 | 5523 |
Azidothymidine | 233 | 0.015 | 15882 |
2',3'-Dideoxycytidine | 2145 | 1.2 | 1789 |

CC₅₀: 50% Cytotoxic concentration; EC₅₀: 50% effective concentration; SI: selectivity index (CC₅₀/EC₅₀).
Figure 2. Correlation coefficient of chemical descriptors and cytotoxicity of piperic acid amides against tumor cells (defined as T) (A) and normal cells (defined as N) (B). The mean (pCC50, i.e., the −log CC50) values for normal cells and tumor cell lines were defined as N and T, respectively. The descriptors used were: a_hyd (Number of hydrophobic atoms), logP(o/w) (Log of the octanol/water partition coefficient), logS (Log of the aqueous solubility), PEOE_VSA_FNEG (Fractional negative van der Waals surface area), PEOE_VSA_FPOS (Fractional positive van der Waals surface area), PEOE_VSA_NEG (Total negative van der Waals surface area), vsurf_EWmin1 (Lowest hydrophilic energy 1), vsurf_HB6 (H-bond donor capacity 6), vsurf_HB7 (H-bond donor capacity 7), vsurf_IW7 (Hydrophilic interaction-energy moment 7), vsurf_W7 (Hydrophilic volume 7).
p<0.05) and total positive polar van der Waals surface area ($r^2=0.425$, $p<0.05$) (Figure 2B).

Tumor selectivity of piperic acid amides (defined by T–N) correlated with the $v_{surf}$ descriptor in standard hydrophilic interaction-energy moment 8 ($v_{surf}$.IW) ($r^2=0.638$, $p<0.005$), the partial equalization of orbital electronegativity in total polar van der Waals surface area ($r^2=0.609$, $p<0.005$), sum of van der Waals surface area in atoms with the partial charge ($r^2=0.583$, $p<0.005$), fractional positive van der Waals surface area ($r^2=0.583$, $p<0.005$),

Figure 3. Correlation coefficient of chemical descriptors and tumor specificity of piperic acid amides, defined as T–N. The descriptors used were: $a_{NO}$ (Number of oxygen atoms), PEOE_VSA_PNEG (Total negative polar van der Waals surface area), PEOE_VSA_POL (Total polar van der Waals surface area), PEOE_VSA_PPOS (Total positive polar van der Waals surface area), PEOE_VSA+4 (Sum of $v_i$ where $q_i$ is in the range [0.20,0.25]; $v_i$ and $q_i$ denote the van der Waals surface area and the partial charge of atom $i$, respectively), $v_{surf}$.IW (Hydrophilic interaction-energy moment 8).
total negative polar van der Waals surface area ($r^2=0.519$, $p<0.01$) and number of oxygen atoms ($r^2=0.467$, $p<0.05$) (Figure 3).

Discussion

The present study demonstrated for the first time that piperine has minor antitumor potential but no anti-HIV activity, and introduction of a catechol moiety [8] significantly enhanced the tumor specificity. We found that TS values determined by two different equations (either D/B or C/A, see Table I) were considerably variable, suggesting the considerable difference in sensitivity of seven cell lines used to the 12 piperlic acid amide derivatives. It is, thus, necessary that we should use more than three cell lines for both normal and tumor cell groups. Based on these experimental data, we performed the QASR analysis using the D/B value.

We could not obtain significant descriptors for T from the quantum chemical approaches. Therefore, with the assistance of descriptors calculated by MOE, a total 330 parameters were searched. We found that many PEOE descriptors, which provide information on electric charge, and vsurf descriptors, which reflect the molecular shape, explain well the cytotoxicity and tumor-selectivity of piperic acid amides. The PEOE method of calculating atomic partial charges (27) is a method in which charge is transferred between bonded atoms until equilibrium. The vsurf descriptors are similar to the VolSurf descriptors (28), and depend on the structure connectivity and conformation. We found good correlation of T with van der Waals surface area (total negative, fractional positive and negative) and hydrophobic property (Figure 2A). N correlated well with hydrophilic interaction-energy moment and energy, H-bond donor capacity and volume, and total positive polar van der Waals surface area (Figure 2B). The tumor selectivity (T−N) correlated well with van der Waals surface area (total polar, positive polar and negative polar), and number of oxygen atoms (Figure 3). Compound [8] had the highest tumor specificity, possibly due to its unique molecular shape and electrostatic interaction, especially its largest PEOE and vsurf descriptors.

Curcumin (diferuloylmethane), a natural compound extracted from Curcuma longa L., has been reported by many investigators to inhibit the proliferation of various tumor cells in culture, prevent carcinogenesis and inhibit the growth of implanted tumors (29). However, the evaluation system used herein for TS demonstrated that curcumin had a very narrow therapeutic window (TS=1.7) (30). Previous attempts to enhance the antitumor potential of curcumin by introducing piperic acid and glycine (31) or demethoxy, bisdemethoxy or piperoyl (32) groups were unsuccessful.

In conclusion, the present study demonstrates there are many chemical descriptors specific to cytotoxicity against normal and tumor cells, and TS. Tumor selectivity was well-correlated with molecular shape and electrostatic interaction. Multivariate statistics with these chemical descriptors may be useful for designing the most favorable compound with higher tumor selectivity.

References


26 http://www.computational-chemistry.co.uk/


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(a) First page including the title of the presented work [not exceeding fifteen (15) words], full names and full postal addresses of all Authors, name of the Author to whom proofs are to be sent, key words, an abbreviated running title, an indication “review”, “clinical”, “epidemiological”, or “experimental” study, and the date of submission. (Note: The order of the Authors is not necessarily indicative of their contribution to the work. Authors may note their individual contribution(s) in the appropriate section(s) of the presented work); (b) Abstract not exceeding 150 words, organized according to the following headings: Background/Aim - Materials and Methods/Patients and Methods - Results - Conclusion; (c) Introduction; (d) Materials and Methods/Patients and Methods; (e) Results; (f) Discussion; (g) Acknowledgements; (h) References. All pages must be numbered consecutively. Footnotes should be avoided. Review articles may follow a different style according to the subject matter and the Author's opinion. Review articles should not exceed 35 pages (approximately 250 words per double-spaced typed page) including all tables, figures, and references.

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