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BRIEF REPORT

Antiviral Susceptibility of Highly Pathogenic Avian Influenza A(H5N1) Viruses Circulating Globally in 2022–2023

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The antiviral susceptibility of currently circulating (2022–2023) highly pathogenic avian influenza (HPAI) A(H5N1) viruses was assessed by genotypic and phenotypic approaches. The frequency was low for neuraminidase (NA) and polymerase acidic (PA) substitutions associated with reduced inhibition by NA inhibitors (21/2698, 0.78%) or the PA inhibitor baloxavir (14/2600, 0.54%). Phenotypic testing of 22 clade 2.3.2.1a and 2.3.4.4b viruses revealed broad susceptibility to NA inhibitors and baloxavir for a conclusion that most contemporary HPAI A(H5N1) viruses retain susceptibility to antiviral drugs. Novel NA-K432E and NA-T438I substitutions (N2 numbering) were identified at elevated frequencies (104/2698, 3.85%) and caused reduced zanamivir and peramivir inhibition.

Keywords. resistance; avian influenza; baloxavir; H5N1; neuraminidase inhibitors; .

This study is a timely antiviral risk assessment of highly pathogenic influenza A(H5N1) viruses (2022–2023). Most viruses screened are genotypically and/or phenotypically susceptible to existing antivirals. Two novel neuraminidase (NA) substitutions that cause reduced inhibition by zanamivir/peramivir have been identified.

Circulation of highly pathogenic avian influenza (HPAI) A(H5N1) viruses constitutes an ongoing burden for the poultry industry and poses a zoonotic public health threat. Since 2020, novel A(H5N1) clade 2.3.4.4b viruses spread from Southeast Asia to Europe and the Americas, causing wild bird and poultry outbreaks. The years 2021 to 2022 witnessed further panzootic spread in diverse wild bird species and sporadically among aquatic and small terrestrial mammals, increasing risk of mammalian adaptation and/or increased transmissibility [1]. According to the World Health Organization, only 14 human A(H5N1) infections occurred from 2022 to 2023, but their wide geographic distribution is concerning. A Food and Drug Administration–approved adjuvated A(H5N1) vaccine is stockpiled in the United States and may cross-react against other clades [2], but insufficient data exist to understand its potential against 2.3.4.4b viruses. Therefore, antivirals can play an important role in virus control and pandemic preparedness. Two classes of direct-acting influenza drugs are available: NA inhibitors (NAIs) and a cap-dependent endonuclease inhibitor (CENI). However, emergence of drug-resistant variants can limit therapeutic options, as occurred with the adamantanes that are now ineffective for seasonal influenza [3]. Therefore, susceptibility to existing antivirals is critical to risk assessment of emerging influenza viruses.

Here, we analyzed the frequency of established NA and polymerase acidic (PA) protein molecular markers associated with reduced or highly reduced inhibition (RI/HRI) by NAIs and baloxavir in HPAI A(H5N1) viruses circulating worldwide in 2022 to 2023. Phenotypic susceptibility was also determined for each drug class.

METHODS

Viruses, Cells, and Compounds

HPAI A(H5N1) viruses were propagated in allantoic cavities of 10-day-old embryonated chicken eggs (40 hours, 35 °C). Recombinant A/bald eagle/Florida/W22-134-OP/2022 (H5N1) NA-T438I (N2 numbering) and wild type hemagglutinin (HA) genes, with 6 internal segments from the A/Puerto Rico/8/1934 (H1N1) virus, were generated by site-directed mutagenesis (Agilent) and reverse genetics [1]. Experiments were conducted under biosafety level 3+ conditions in compliance with applicable laws and guidance. Madin-Darby canine kidney (MDCK) cells and human embryonic kidney (HEK293T) cells were obtained from ATCC and maintained according to manufacturer instructions. NAIs and baloxavir acid were purchased from MedChem Express.

NAI Susceptibility Determination

Susceptibility to NAIs (oseltamivir carboxylate [oseltamivir], peramivir, and zanamivir) was assessed with a fluorogenic assay with substrate MUNANA (2′−[4-methylumbelliferyl]-α-D-N-acetylneuraminic acid; Sigma-Aldrich) [4]. NA activity of each virus was standardized to relative fluorescent unit equivalents of 10 μM 4-methylumbelliferone. Fluorescent NA-cleaved

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substrate was measured with a BioTek Multimode Plate Reader (Agilent) at Ex/Em 360 and 460 nm. The half-maximal inhibitory concentration (IC_{50}) of the NAI was calculated with Prism (version 9.5; GraphPad) via a sigmoidal dose-response equation (variable slope).

CENI Susceptibility Determination

CENI susceptibility was assessed in MDCK cells by IRINA (influenza replication inhibition NA-based assay), with virus inoculum normalized to 1.9 nM/well of 4-methylumbelliferone [4]. Inocula were incubated with baloxavir (0.006–111 nM) and MDCK cells in 96-well microplates (24 hours, 37 °C). NA activity and baloxavir EC_{50} (half-maximal effective concentration) were calculated as described for NAI assays.

Gene Sequencing and Analysis

Viral RNA was extracted (RNeasy; Qiagen) and cDNA synthesized (Superscript III Reverse Transcriptase Kit; Life Technologies). Virus genome segments were amplified (Phusion High-Fidelity DNA Polymerase; New England Biolabs) with universal conserved Uni12/13 primers and a QIAquick PCR Purification Kit (Qiagen). Deep sequencing (Nextera XT; Illumina) was performed by the Hartwell Center at St. Jude Children's Research Hospital (St. Jude), and NA, PA, and MP gene sequences of 203 viruses were uploaded to GenBank (Supplementary Table 1). These and publicly available sequences from GenBank and GISAID (Global Initiative on Sharing All Influenza Data) underwent alignments via progressive (FFT-NS-2) and iterative (FFT-NS-i) refinement by MAFFT (version 7) with amino acid variant calling in BioEdit (version 7.7.1).

RESULTS

Genotypic Analysis of HPAI A(H5N1) Viruses

Frequency of NA substitutions associated with NAI RI/HRI was determined from 2698 global HPAI A(H5N1) sequences (Table 1). The analysis was based on NA markers reported previously [5]. No RI/HRI-associated NA substitutions were identified in viruses from the African, South American, or Oceanic regions, whereas RI/HRI-associated substitutions were identified at frequencies of 2.05%, 0.68%, and 0.76% in Asian, European, and North American (NAm) viruses, respectively. NA-I117T mediates oseltamivir/zanamivir RI and was identified in 8 viruses: 1 Asian, 6 European, and 1 NAm. NA-S246N mediates oseltamivir RI and was identified in 2 NAm viruses. NA-H274Y mediates oseltamivir/peramivir RI/HRI in A(H5N1) viruses and was identified in 4 NAm viruses. One Asian virus had NA-R292K, which causes oseltamivir RI

Table 1. Frequency of HPAI A(H5N1) Viruses Circulating Globally in 2022–2023 and Carrying Substitutions Associated With RI/HRI by NAIs and/or CENI

	HPAI A(H5N1) Viruses With NA or PA Substitutions, No. (%)[a]			
NA or PA Substitution[b]	Asia	Europe	North America	Worldwide
NAI RI/HRI associated				
NA-I117T	1 (0.69)	6 (0.51)	1 (0.08)	8 (0.30)
NA-R152K	0	1 (0.09)	0	1 (0.04)
NA-S246N	0	0	2 (0.15)	2 (0.07)
NA-H274Y	0	0	4 (0.30)	4 (0.15)
NA-R292K	1 (0.69)	0	0	1 (0.04)
NA-N294S	1 (0.69)	1 (0.09)	3 (0.23)	5 (0.19)
Total	3/146 (2.05)	8/1175 (0.68)	10/1320 (0.76)	21/2698 (0.78)
Potential NAI RI/HRI associated				
NA-K432E	0	56 (4.77)	1 (0.08)	57 (2.12)
NA-T438I	0	0	47 (3.56)	47 (1.75)
Total	0/146	56/1175 (4.77)	48/1320 (3.64)	104/2698 (3.85)
CENI RI associated				
PA-E23K	0	1 (0.09)	0	1 (0.04)
PA-K34R	0	0	1 (0.08)	1 (0.04)
PA-A36V	0	0	1 (0.08)	1 (0.04)
PA-A37T	0	0	4 (0.32)	4 (0.15)
PA-I38M	0	0	1 (0.08)	1 (0.04)
PA-I38T	0	0	2 (0.16)	2 (0.08)
PA-E199G	0	4 (0.35)	0	4 (0.15)
Total	0/119	5/1154 (0.43)	9/1270 (0.71)	14/2600 (0.54)

Abbreviations: CENI, cap-dependent endonuclease inhibitor; HPAI, highly pathogenic avian influenza; HRI, highly reduced inhibition; NA, neuraminidase; NAI, neuraminidase inhibitor; PA, polymerase acidic; RI, reduced inhibition.

[a]Publicly available NA and PA sequences from global HPAI A(H5N1) viruses (week 1, 2022–week 22, 2023) were retrieved from GISAID (Global Initiative on Sharing All Influenza Data) and the National Center for Biotechnology Information's Influenza Virus Sequence Database (accessed 1 May 2023). Duplicated virus sequences were removed by the SeqKit platform for FASTA/Q file manipulations (https://github.com/shenwei356/seqkit).

[b]Amino acid residue substitutions in the NA protein are indicated by N2 numbering and those in the PA protein by influenza A virus numbering.

in A(H1N1)pdm09 viruses [6]. NA-N294S mediates oseltamivir/zanamivir/peramivir RI/HRI and was identified in 1 Asian, 1 European, and 3 NAm viruses. Several established RI/HRI-associated NA substitutions were not found in currently circulating A(H5N1) viruses of clades 2.3.2.1a and 2.3.4.4b (NA-V116A, NA-E119A/D/G, NA-Q136L, NA-D198G, NA-I222M/V, and NA-K432T). Overall, the frequency of HPAI A(H5N1) viruses with known NAI RI/HRI–associated substitutions was low (0.78%). Conversely, there was a higher frequency of the novel NA-K432E substitution in European 2.3.4.4b viruses (4.77%) and the novel NA-T438I substitution in NAm 2.3.4.4b viruses (3.56%).

Frequency of PA substitutions associated with CENI RI was determined from 2600 global HPAI A(H5N1) sequences (Table 1). The analysis was based on PA markers reported previously [5]. No CENI RI–associated markers were identified in viruses from Asian, African, South American, or Oceanic regions. CENI RI–associated PA markers were identified at frequencies of 0.43% in European viruses and 0.71% in NAm viruses. Substitutions at PA-I38X cause the most pronounced CENI RI [3]. Two NAm viruses had PA-I38T and 1 had PA-I38M. PA-E23X substitutions cause less severe CENI RI, and PA-E23K was identified in 1 European virus. Other substitutions, including PA-K34R, PA-A36V, and PA-A37T, may cause subtype-specific CENI RI [5], and these were identified in 1, 1, and 4 NAm viruses, respectively. Similarly, PA-E199G may mediate subtype-conditional CENI RI and was present in 4 European viruses. Overall, frequency of HPAI A(H5N1) viruses with well-described CENI RI–associated PA substitutions was low (0.54%). Highly pathogenic (HP) A(H5N1) viruses isolated from mammals and humans in 2022-2023 did not carry any of the previosly reported NAI/CENI RI/HRI-associated substitutions (Supplementary Table 2).

Phenotypic Analysis of HPAI A(H5N1) Viruses

Using a panel of viruses available from the St. Jude repository, we assessed NAI susceptibilities of the predominant circulating clades 2.3.2.1a and 2.3.4.4b with a fluorescence-based NA inhibition assay (Table 2). Median IC_{50} values were nanomolar concentrations for oseltamivir (1.05–1.46 nM) and subnanomolar concentrations for zanamivir (0.24–0.32 nM) and peramivir (0.09–0.21 nM). The IC_{50} values for oseltamivir were 3- to 6-fold higher than zanamivir and 5- to 16-fold higher than peramivir. NAm clade 2.3.4.4b viruses were slightly more susceptible to peramivir than Asian 2.3.2.1a viruses. Notably, oseltamivir IC_{50} values were higher for HPAI A(H5N1) viruses than for seasonal human A(H1N1)pdm09 and A(H3N2) reference viruses and/or past surveillance reports [7]. A virus with NA-S246N displayed a 6-fold increase in oseltamivir IC_{50} and 5-fold increase in peramivir IC_{50} as compared with the median 2.3.4.4.b A(H5N1) reference, indicating normal inhibition. Previously unreported NA-K432E in A/bald eagle/Virginia/W23-101/2023 conferred 8-, 23-, and 7-fold RI by oseltamivir, zanamivir, and peramivir, respectively (Table 2). Seven viruses containing a previously unreported NA-T438I substitution displayed RI by zanamivir (IC_{50}, 4.18–23.81 nM) when compared with baseline values of clade 2.3.4.4b A(H5N1) and A(H1N1) pdm09 viruses. Median ± SD IC_{50} (0.86 ± 0.28 nM) by peramivir for NA-T438I viruses represents a 10-fold increase over baseline (0.09 ± 0.01 nM), indicating RI. To confirm the effect of NA-T438I on NAI susceptibility, we generated recombinant NA-T438I A/bald eagle/Florida/W22-134OP/2022 virus, which had a zanamivir IC_{50} of 8.89 nM, 47-fold the recombinant wild type virus IC_{50} or 37-fold the 2.3.4.4.b median IC_{50} values. Peramivir IC_{50} was 1.12 nM, corresponding to a 10- to 12-fold increase as compared with rg-A/bald eagle/Florida/W22-134OP/2022 and the 2.3.4.4b median IC_{50} values. Notably, A/bald eagle/Tennessee/W23-003/2023 virus with NA-T438I had previously unreported NA-K143R/NA-T213M substitutions, and the observed inhibition approached RI with oseltamivir (8-fold) and HRI with zanamivir (98-fold). Four other NA-T438I viruses with zanamivir RI (>30-fold) contained additional NA-I288V/NA-M289V substitutions. The degree to which these non-T438I substitutions influence NAI susceptibility is unknown and warrants further study.

The phenotypic CENI susceptibility of A(H5N1) viruses was assessed with an MDCK cell–based IRINA (Table 2). Median EC_{50} values for both clades were subnanomolar (2.3.2.1a, 0.30 nM; 2.3.4.4b, 0.29 nM). Clade 2.3.4.4b viruses carrying NA-T438I, NA-K143R, or NA-T213M did not show RI by baloxavir [8].

DISCUSSION

An essential step in the risk assessment of emerging influenza viruses is determining susceptibility to available antivirals. Here, we conducted genotypic and phenotypic screenings of HPAI A(H5N1) viruses circulating globally in 2022 to 2023. Both screening techniques indicated that these 2 most predominant clades are largely susceptible to NAIs and CENI. The incidence of well-described NAI or CENI RI/HRI markers was <1% and is supported by a recent study of NAm 2.3.4.4b viruses showing 0.8% incidence of RI [8]. We observed the absence of NA-E119A, NA-E119D, and NA-E119G, which have variable negative impacts on NAI susceptibility [5]. HPAI A(H5N1) viruses with NA-H274Y, NA-R292K, NA-N294S, NA-R152K, or PA substitutions were unavailable for phenotypic testing, but the literature suggests that they will have decreased drug susceptibility [5]. One of 2 viruses containing NA-S246N was available and demonstrated a 6-fold increase in IC_{50} for the median clade-specific values, failing to meet RI criteria. In contrast, viruses in Southeast Asia from 2006 to 2008 displayed oseltamivir RI [9]. IC_{50} values were compared with seasonal human A(H1N1) reference IC_{50} values, which

Table 2. NAI and CENI Susceptibility of Clade 2.3.2.1a and 2.3.4.4b HPAI A(H5N1) Viruses Circulating Globally in 2022–2023

		Susceptibility to Antiviral Drugs, nM, Mean ± SD (Fold Change)			
		NAI, IC_{50}			CENI, EC_{50}
HA Clade of HPAI A(H5N1) Virus/Reference Virus	Amino Acid Substitution[a]	Oseltamivir	Zanamivir	Peramivir	Baloxavir [b]
Clade 2.3.2.1a					
Median (n = 10)	—	1.05 ± 0.52	0.32 ± 0.08	0.21 ± 0.03	0.30 ± 0.06
Clade 2.3.4.4b					
Median (n = 12)[c]	—	1.46 ± 0.18	0.24 ± 0.08	0.09 ± 0.01	0.29 ± 0.06
A/red-tailed hawk/Kansas/W23-216/2023	NA-S246N	8.65 ± 0.24 (6)	0.21 ± 0.02 (1)	0.48 ± 0.07 (5)	0.92 ± 0.11 (3)
A/bald eagle/Virginia/W23-101/2023	NA-K432E	11.75 ± 2.08 (8)	5.52 ± 0.89 (23)	0.63 ± 0.23 (7)	0.18 ± 0.01 (1)
A/black vulture/Georgia/W22-933C/2022	NA-T438I	3.95 ± 0.56 (3)	13.01 ± 1.62 (54)	1.41 ± 0.67 (16)	0.60 ± 0.11 (2)
A/black vulture/North Carolina/W22-1079C/2022	NA-T438I	2.96 ± 0.30 (2)	7.24 ± 0.86 (30)	1.10 ± 0.29 (12)	0.26 ± 0.05 (1)
A/black vulture/South Carolina/W22-1080A/2022	NA-T438I	2.09 ± 0.34 (1)	4.18 ± 0.16 (17)	0.50 ± 0.13 (6)	0.33 ± 0.13 (1)
A/bald eagle/Tennessee/W23-003/2023	NA-T438I	11.35 ± 1.10 (8)	23.81 ± 5.17 (98)	2.06 ± 0.52 (23)	0.80 ± 0.06 (3)
A/black vulture/Louisiana/W23-118/2023	NA-T438I	3.39 ± 0.23 (2)	7.10 ± 0.72 (30)	0.66 ± 0.18 (7)	0.50 ± 0.12 (2)
A/bald eagle/South Carolina/W23-147/2023	NA-T438I	7.96 ± 0.42 (5)	9.31 ± 2.08 (38)	0.86 ± 0.28 (10)	0.27 ± 0.02 (1)
A/black vulture/Louisiana/W23-166/2023	NA-T438I	6.62 ± 0.45 (4)	9.81 ± 1.74 (40)	0.85 ± 0.25 (9)	0.33 ± 0.07 (1)
Median (n = 7)	NA-T438I	3.95 ± 0.42 (3)	9.31 ± 1.62 (39)	0.86 ± 0.28 (10)	0.33 ± 0.07 (1)
rg-A/bald eagle/Florida/W22-134-OP/2022	—	1.22 ± 0.14	0.19 ± 0.01	0.11 ± 0.02	0.53 ± 0.14
rg-A/bald eagle/Florida/W22-134-OP/2022	NA-T438I	6.44 ± 0.19 (5)	8.89 ± 2.43 (47)	1.12 ± 0.39 (10)	1.50 ± 0.37 (3)
Reference A(H1N1)pdm09					
A/Denmark/524/2009	—	0.37 ± 0.25	0.31 ± 0.17	0.09 ± 0.01	N/A
A/Denmark/528/2009	NA-H274Y	240.88 ± 10.36 (651)	0.40 ± 0.38 (1)	29.00 ± 2.63 (322)	N/A
rg-A/California/04/2009	—	N/A	N/A	N/A	0.61 ± 0.06
rg-A/California/04/2009	PA-I38T	N/A	N/A	N/A	23.24 ± 2.94 (38)

Data are presented from 2 independent experiments. As recommended by the World Health Organization Expert Working Group on Antiviral Susceptibility (in support of the Global Influenza Surveillance and Response System), the inhibition thresholds (cutoff) for NAIs are as follows: normal inhibition for influenza A viruses, <10-fold; reduced inhibition, 10- to 100-fold; highly reduced inhibition, >100-fold [1]. An arbitrary threshold of a ≥3-fold increase in median EC_{50} is used for reporting influenza A and B viruses with reduced inhibition by baloxavir [5].

Abbreviations: —, amino acid substitution not present; CENI, cap-dependent endonuclease inhibitor; EC_{50}, half-maximal effective concentration; HA, hemagglutinin; HPAI, highly pathogenic avian influenza; IC_{50}, half-maximal inhibitory concentration; N/A, not applicable; NA, neuraminidase; NAI, neuraminidase inhibitor; PA, polymerase acidic; rg, recombinant virus.

[a]Amino acid residue substitutions in the NA protein are indicated by N2 numbering.

[b]Baloxavir acid: the active metabolite form of the prodrug baloxavir marboxil.

[c]A panel of 12 clade 2.3.4.4b A(H5N1) viruses lacking the amino acid substitutions in the NA head domain (residues 82–470) and in the PA endonuclease active site (residues 1–200) was used to determine the median IC_{50}/EC_{50} (ie, the baseline susceptibility; data presented in Supplementary Table 3). A fold change in IC_{50} and EC_{50} values for viruses carrying the designated amino acid substitutions was calculated relative to the baseline susceptibility. For rg-A/bald eagle/Florida/W22-134-OP/2022 and human influenza viruses, IC_{50} and EC_{50} fold changes were determined by comparisons with NA/PA sequence–matched wild type reference viruses.

may partly account for the discrepancy. Global frequency of HP A(H5N1) viruses isolated from birds and mammals in 2022 to 2023 and carrying adamantane RI substitutions remains relatively low (3.3%–3.4%) as compared with circulating human seasonal viruses (>99%). However, human A(H5N1) infections in Cambodia did contain adamantane resistance marker M2-S31N (Supplementary Table 4).

In this study, the median IC_{50} values for zanamivir and baloxavir were similar to those for human A(H1N1)pdm09 and A(H3N2) viruses (≤2-fold change), whereas the oseltamivir IC_{50} values were 6-fold higher. Similar findings were reported for other A(H5N1) clades [10, 11], suggesting that an A(H5N1) reference is required for proper RI/HRI determination. Our data begin to address this discrepancy by providing median IC_{50} values specific to the A(H5N1) subtype and specifically for isolates from the avian reservoir.

Another critical finding is that previously unreported NA-K432E and NA-T438I substitutions mediate zanamivir and peramivir RI in clade 2.3.4.4b A(H5N1) viruses. NA-T438X substitutions are poorly described with respect to NAI susceptibility. However, in N1 and N4 subtypes, substitutions emerged nearby at NA-I427L and NA-I436N under zanamivir pressure, causing RI in vitro [12, 13]. Additionally, NA-K/Q432 is relatively conserved among influenza A and B viruses, stabilizing the structurally significant NA-430 loop [14], and NA-K432T caused zanamivir RI in clade 1 A(H5N1) viruses [10]. A study of NAm 2.3.4.4b viruses recently identified that NA-T438N caused 12-fold reduced zanamivir IC_{50} values, while NA-T438N + N294S synergized to reduce the susceptibility of all NAIs [8]. Our data show that NA-K432E, highly prevalent in European 2.3.4.4b viruses, also confers zanamivir RI. Additionally, NA-T438N viruses may pose a heightened risk, as this was the most frequent NA substitution in our genotypic analysis and mediated zanamivir RI/HRI. Whether NA-T438I or other substitutions at this position are zanamivir and peramivir RI marker specific to avian viruses

or applicable to human viruses or non-A(H5N1) subtypes is unknown. Identification of novel substitutions would ideally include sequence-based [5] and phenotypic confirmation through functional assays and/or generation of recombinant viruses as permitted by current regulations. Additionally, fitness-based assays including replication kinetics and in vivo models should be incorporated. Importantly, NA-T438I viruses do have poorer NA enzyme activity vs wild type, despite overall productive titers from tissue culture inoculations [8]. The mechanisms by which viruses with NAI-associated substitutions appear in avian species requires further study, but waterfowl exposure to NAIs is possible due to the excreted active metabolite oseltamivir carboxylate not being removed during traditional sewage treatment [15].

As clade 2.3.4.4b A(H5N1) viruses continue their epizootic expansion, our study represents a timely assessment of their risk to human health and provides insight into the efficacy of antiviral interventions. With few exceptions, the viruses screened were genotypically and/or phenotypically susceptible to most NAIs, suggesting that current antivirals could be successful adjunct treatments for human infections. However, the elevated frequency of NA-K432E and NA-T438I may challenge the efficacy of some Food and Drug Administration–approved antivirals, particularly zanamivir, although current US antiviral stockpiles rely on oseltamivir. Approval of intravenous zanamivir in the European Union for use under exceptional circumstances and drug availability on compassionate use in some countries could extend pandemic use.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copy-edited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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