Chimeric oligonucleotides based on 2′-*O*-modified oligoribonucleotides with the terminal 3′—3′ internucleotide linkage as potential inhibitors of *MDR 1* gene expression

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Chimeric constructs were synthesized based on oligoribonucleotides modified at the 2′-position of the ribose (2′-*O*-tetrahydropyranyl- or 2′-*O*-methyl-) and at the 3′-terminus of the oligonucleotide chain (terminal 3′—3′ internucleotide linkage), which are complementary to a region of *MDR 1* mRNA. A comparative study of the properties of these chimeric constructs was performed. The chimeric oligomers with the modified 3′-terminus are characterized by high stability with respect to 3′-exonucleases, form stable complementary complexes with RNA, and can activate RNase H in a duplex with RNA.

Key words: chimeric oligonucleotides, 2′-*O*-modified oligoribonucleotides, multi-drug resistance gene, mRNA, antisense approach, thermal stability, RNase H, stability, nucleases.

Synthetic oligonucleotides serve as a powerful tool for the selective action on nucleic acids. These compounds can inhibit expression of particular genes *via* the complementary recognition of regions of the coding (sense) mRNA sequence and the corresponding blocking of its functions through the physical arrest or degradation by RNase H.[1] The multi-drug resistance gene *MDR 1* is responsible for cross stability of tumor cells with respect to a broad spectrum of chemiotherapeutic drugs due to which these agents cannot be used for the treatments of tumor diseases.[2] Inhibition of expression of this gene is a topical problem of modern molecular biology and medicine.

In biological media, natural oligonucleotides undergo rapid cleavage. Because of this, the use of various modified oligonucleotides for the above-mentioned purpose has attracted considerable recent attention. One line of investigation involves the design of minimum modified oligonucleotides possessing a number of improved biological and physicochemical characteristics. Among these compounds, analogs of oligoribonucleotides, which combine modifications at both the 2′-*O* position of the ribose and the 3′-terminus of the oligonucleotide chain, are of particular interest. These oligomers can form stable duplexes with RNA and possess enhanced stability with respect to endo- and exonucleases.[1,3,4] Since RNA duplexes with 2′-*O*-modified oligonucleotides are not recognized by RNase H,[5] it was proposed that chimeric constructs containing the central oligodeoxyribo fragment should be designed.[6,7] The presence of this fragment provides activation of RNase H.

In the present study, we synthesized hexadecamers and performed comparative analysis of their properties. These hexadecamers represent the central oligodeoxyribo fragment and wings composed of the 2′-*O*-modified (2′-*O*-tetrahydropyranyl- or 2′-*O*-methyl-) oligoribo fragments with the terminal 3′—3′ internucleotide linkage, which are complementary to a region of *MDR 1* mRNA.

Experimental

Nucleosides and reagents were either produced at Russian plants or were purchased from Fluka (Switzerland), Merck (Germany), and Sigma (USA). The following enzymes were used: *Penicillium citrinum* nuclease P_1 (EC 3.1.30.1; 100 IU mL^{-1}; Sigma, USA), *E.coli* alkaline phosphatase (EC 3.1.3.1; 34 IU mg^{-1}; NPO Biolar, Latvia), *Crotalus atros venom* phosphodiesterase (SVP; EC 3.1.4.1; 0.9 IU mL^{-1}; Sigma, USA), T4 polynucleotide kinase (EC 2.7.1.78; 5000 IU mL^{-1}; Sibenzim, Russia), and *E.coli* RNase H (EC 3.1.4.34; Hybaid, UK). The octadecaribonucleotide 5′-UCCAAGGAGCGCGUGGUC was kindly supplied by M. N. Repkova (Novosibirsk Institute of Bioorganic Chemistry, Siberian Division of the Russian Academy of Sciences).

Preparation of a polymeric support with bound 3′-*O*-dimethoxytritylthymidine was carried out as described previously.[8] The capacity of the nucleoside-bound polymer thus obtained was 28 μmol g^{-1}.

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Synthesis of 2′-*O*-modified oligoribonucleotides and their analogs. Oligonucleotides were synthesized by the solid-phase H-phosphonate method in the manual mode.[9—11] The oligomers were isolated by ion-exchange and reversed-phase (RP) HPLC on a Waters liquid chromatograph (USA) or by electrophoresis in 20% polyacrylamide gel (PAAG) under denaturating conditions.[12] The structures of the resulting oligonucleotides were confirmed by exhaustive enzymatic hydrolysis with a mixture of SVP and alkaline phosphatase or (in the case of 3′—3′-inverted analogs) with nuclease P_1 and alkaline phosphatase followed by quantitative analysis of the hydrolyzate by RP HPLC. The 2′-*O*-tetrahydropyranyl-containing oligomers were preliminarily subjected to acid hydrolysis[13] to remove 2′-*O*-protective groups.

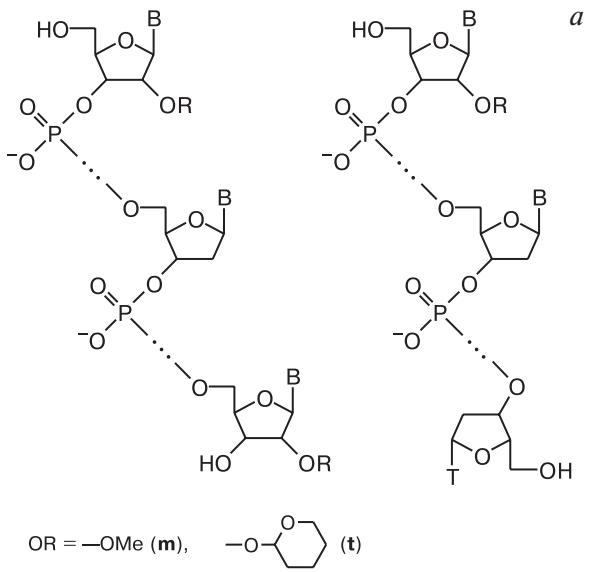
Introduction of a radioactive label at the 5′-terminus of the oligomers was carried out according to a known procedure.[14]

Study of stability of oligonucleotides with respect to SVP. A solution (2 μL) of $2 \cdot 10^{-4}$ IU SVP in a buffer (10 *mM* tris(hydroxymethyl)aminomethane (Tris)—HCl, pH 7.8, 0.5 *mM* $MgCl_2$) was added to a solution of a 5′-[^{32}P]-labeled oligonucleotide (10^{-5} mol L^{-1}) in the same buffer (18 μL) and the mixture was incubated at 37 °C. Aliquots were taken at certain intervals. The nucleotide material was precipitated with a 2% $LiClO_4$ solution in acetone and analyzed by gel electrophoresis followed by autoradiography. The autoradiograph was digitized with the use of the Gel-Pro Analyzer program package (Media Cybernetics, Inc., USA) and then the content of the starting oligomer in the specimen was calculated as a ratio of the peak area of the starting oligomer to the sum of the peak areas of the degradation products and the starting oligomer. The relative error of the determination was no higher than 20%. The results are presented in Fig. 1.

Hydrolysis of RNA with *E.coli* RNase H was carried out in a buffer (20 *mM* 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)—KOH, pH 8.0, 4 *mM* $MgCl_2$, 5 *mM* KCl, 0.05% bovine serum albumin) containing a fragment of *MDR 1* mRNA (10^{-7} mol L^{-1}) and the complementary oligonucleotide (10^{-5} mol L^{-1}). The reaction mixtures (10 μL) were incubated at 20 °C for 15 min and then 0.01 IU RNase H was added. At certain intervals, the reaction mixtures were precipitated with a 2% $LiClO_4$ solution in acetone and applied onto 20% denaturating PAAG for electrophoretic analysis. The autoradiograph was processed as described above. The degree of RNA cleavage was determined as the ratio between the sum of the peak areas corresponding to the cleavage products and the sum of the peak areas of the starting oligomer and cleavage products. The results are shown in Fig. 2.

Limited hydrolysis of the synthetic fragment of *MDR 1* mRNA with SVP. A solution (0.5 μL) of $1 \cdot 10^{-4}$ IU SVP in a buffer (10 *mM* Tris—HCl, pH 7.8, 0.5 *mM* $MgCl_2$) was added to a solution (5 μL) of a 5′-[^{32}P]-labeled octadecaribonucleotide in the same buffer. The mixture was incubated at 37 °C for 5 min and applied onto 20% denaturating PAAG as a check for the identification of the sites of RNA cleavage by RNase H.

Thermal denaturation curves for the oligonucleotide complexes were recorded in a buffer containing 100 *mM* NaCl, 10 *mM* sodium cacodylate (pH 6.0), and 5 *mM* $MgCl_2$ at a rate of heating of 0.1 °C min^{-1} at the equimolar RNA target : oligomer ratio (the total concentration in the solution



5′-$G^mA^mC^mC^mU^mC^mG^mC^mG^mC^mU^mC^mC^mU^mU^m$	(m_{15})
5′-$G^mA^mC^mC^mU^mC^mG^mC^mG^mC^mU^mC^mC^mU^mU^m{}_{3'-3'}T$	(m_{15}-inv)
5′-$G^tA^tC^tC^tU^tC^tG^tC^tG^tC^tU^tC^tC^tU^tU^t$	(t_{15})
5′-$G^tA^tC^tC^tU^tC^tG^tC^tG^tC^tU^tC^tC^tU^tU^t{}_{3'-3'}T$	(t_{15}-inv)
5′-$G^mA^mC^m$d(CTCGCGCT)$C^mC^mU^mU^m$	($m_3d_8m_4$)
5′-$G^mA^mC^m$d(CTCGCGCT)$C^mC^mU^mU^m{}_{3'-3'}T$	($m_3d_8m_4$-inv)
5′-$G^tA^tC^t$d(CTCGCGCT)$C^tC^tU^tU^t$	($t_3d_8t_4$)
5′-$G^tA^tC^t$d(CTCGCGCT)$C^tC^tU^tU^t{}_{3'-3'}T$	($t_3d_8t_4$-inv)
5′-d(GACCTCGCGCTCCTT)	(d_{15})
5′-d(GACCTCGCGCTCCTT$_{3'-3'}$T)	(d_{15}-inv)

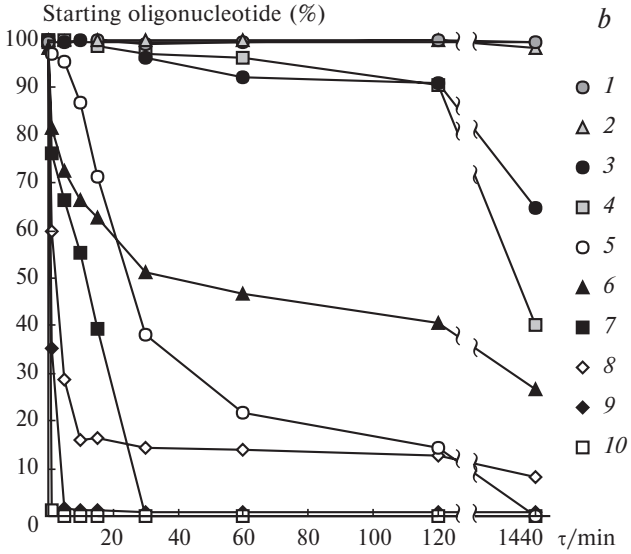


Fig. 1. Oligonucleotides (*a*) and kinetic curves (*b*) of degradation of chimeric oligonucleotides and their 2′-*O*-modified and deoxyribo analogs with SVP (0.01 IU · mL^{-1}, 10 *mM* Tris—HCl, pH 7.8, 0.5 *mM* $MgCl_2$, 37 °C); the concentration of the oligonucleotides was $1 \cdot 10^{-5}$ mol L^{-1}: *1*, t_{15}-inv; *2*, m_{15}-inv; *3*, $t_3d_8t_4$-inv; *4*, $m_3d_8m_4$-inv; *5*, d_{15}-inv; *6*, t_{15}; *7*, $t_3d_8t_4$; *8*, m_{15}; *9*, $m_3d_8m_4$; *10*, d_{15}.

a

5'-U C C A A G G **A G C G C G A G** G U C
3'-$U^R U^R C^R C^R$**T C G C G C T C** $C^R A^R G^R$ (**$t_3d_8t_4$, $m_3d_8m_4$**)

5'-U C C A A G G **A G C G C G A G** G U C
3'-$T_{3'-3'}$,$U^R U^R C^R C^R$**T C G C G C T C** $C^R A^R G^R$ (**$t_3d_8t_4$-inv, $m_3d_8m_4$-inv**)

R = —OMe (**m**), —O— (**t**)

5'-U C C **A A G G A G C G C G A G G U C**
3'-**T T C C T C G C G C T C C A G** (**d_{15}**)

5'-U C C **A A G G A G C G C G A G G U C**
3'-$T_{3'-3'}$,**T T C C T C G C G C T C C A G** (**d_{15}-inv**)

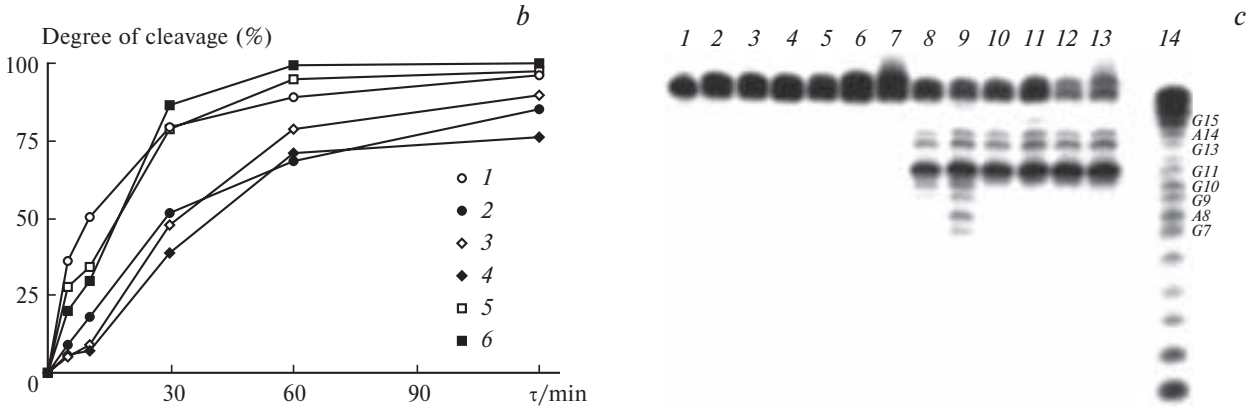


Fig. 2. Cleavage of duplex RNA (*a*) by RNase H. The reaction was carried out in a buffer (20 *mM* HEPES—KOH, pH 8.0, 4 *mM* $MgCl_2$, 5 *mM* KCl, 0.05% bovine serum albumin) containing 0.015 IU *E.coli* RNase H, target RNA (10^{-7} mol L^{-1}), and the oligomer (10^{-5} mol L^{-1}) at 37 °C. *b*. The kinetic curves for RNA cleavage: *1*, d_{15}; *2*, d_{15}-inv; *3*, $m_3d_8m_4$; *4*, $m_3d_8m_4$-inv; *5*, $t_3d_8t_4$; *6*, $t_3d_8t_4$-inv. *c*. RNA cleavage sites: *1*, target RNA in the buffer; *2—7*, specimens without RNase H; *8—13*, specimens incubated in the presence of RNase H for 30 min; *2* and *8*, target RNA+d_{15}; *3* and *9,* target RNA+d_{15}-inv; *4* and *10*, target RNA+$t_3d_8t_4$; *5* and *11*, target RNA+$t_3d_8t_4$-inv; *6* and *12*, target RNA+$m_3d_8m_4$; *7* and *13*, target RNA+$m_3d_8m_4$-inv; *14*, limited hydrolysis of target RNA with SVP (the conditions are given in the Experimental section).

was $5 \cdot 10^{-6}$ mol L^{-1}). The change in the optical absorption of solutions of the complexes upon their heating in a 560-μL cell was followed at two wavelengths (260 and 580 nm) on a Uvikon spectrophotometer (Kontron Instruments, France). The differential curves were obtained from the integral curves by calculating an increase in the optical density as the temperature was changed by 1 °C (Table 1).

Table 1. Melting points of the complexes of the chimeric oligonucleotides and their deoxyribo, 2′-*O*-methylribo, and 2′-*O*-tetrahydropyranylribo analogs with the oligoribonucleotide UCCAAGGAGCGCGAGGUC (region 122—137 of *MDR 1* mRNA)

Oligomer	T_m/°C	Oligomer	T_m/°C
d_{15}	75.9	m_{15}-inv	85.0
d_{15}-inv	75.8	$t_3d_8t_4$	74.7
t_{15}	78.5	$t_3d_8t_4$-inv	75.6
t_{15}-inv	79.4	$m_3d_8m_4$	77.6
m_{15}	85.0	$m_3d_8m_4$-inv	79.5

Results and Discussion

The 2′-*O*-modified oligoribonucleotides and their analogs (see Fig. 1) were synthesized by the solid-phase H-phosphonate method.[9—11] After two preparative HPLCs or preparative gel electrophoresis, the total yield was 5—18%. The oligomers containing inverted thymidine at the 3′-terminus were prepared with the use of polymer-bound 3′-*O*-dimethoxytritylthymidine.[4,15]

Based on the data on stability of short 2′-*O*-modified oligomers with respect to nucleases obtained by us previously,[3] we expected that the 2′-*O*-tetrahydropyranyl- or 2′-*O*-methyl-containing wings of chimeric oligonucleotides in combination with 3′-inverted thymidine would

substantially enhance the resistance of these oligomers to nucleolytic degradation.

The stability of the resulting oligomers with respect to 3′-exonucleases was studied using snake venom phosphodiesterase SVP as an example (see Fig. 1). Pentadecadeoxyribonucleotide was completely degraded within 5 min after the addition of the enzyme, whereas 20% of its 3′-inverted analog remained intact after incubation with the enzyme for 2 h. After 2 h, the degree of degradation of the chimeric oligomers containing inverted thymidine at the 3′-terminus was only 10%, whereas the 2′-*O*-modified oligoribonucleotides with the modified 3′-terminus were stable with respect to SVP during one day. Hence, the introduction of inverted thymidine at the 3′-terminus of oligonucleotides always led to the enhancement of stability of the oligomers.

The degrees of cleavage of target RNA by RNase H in the hybrid complexes with the chimeric oligomers were examined with the use of the synthetic octadecaribonucleotide whose sequence corresponds to the fragment of *MDR 1* mRNA (region 122—137) (see Fig. 2). This region of mRNA was chosen with consideration for the data on the structures and the regions accessible to hybridization.[16,17] Initially, stabilities of the duplexes of the chimeric constructs with target RNA in relation to their deoxyribo, 2′-*O*-tetrahydropyranylribo, and 2′-*O*-methylribo analogs were examined by thermal denaturation (see Table 1). All the complexes under study possess high thermal stability (T_m ranges from 75 to 85 °C), the RNA duplexes with the 2′-*O*-methylated oligomers being most stable. Both in the case of the chimeric constructs and 2′-*O*-modified oligoribonucleotides, the introduction of inverted thymidine at the 3′-terminus did not lead to a decrease in thermal stability of the duplexes (in some cases, thermal stability was even increased).

In complexes with target RNA, the 2′-*O*-modified chimeric constructs induce highly efficient RNA hydrolysis by RNase H (75—100% for 2 h), whereas RNA in the complex with DNA is cleaved by 85—95%. It should be noted that the introduction of inverted thymidine at the 3′-terminus of the 2′-*O*-methylated chimeric oligomers leads to a decrease in the degree of RNA cleavage from 92 to 75%, whereas the 2′-*O*-tetrahydropyranyl-containing chimeric nucleotide and its 3′-modified analog equally efficiently induce RNA hydrolysis. Apparently, the observed differences in the efficiency of hydrolysis of target RNA involved in these heteroduplexes under the action of RNase H are associated with the effect of particular differences in the structure and conformation of the complexes. In the case of the chimeric oligonucleotides and their deoxyribo analogs, the specificity of RNA cleavage proved to be virtually identical. As can be seen from Fig. 2, *c*, the phosphodiester bond between the residues G11 and C10 is the major cleavage site. The fact that the autoradiorgaph has an additional band above target RNA is attributable to high stability of the RNA duplexes under study (see Table 1) and, consequently, to only partial destruction of these duplexes under the standard denaturating conditions of gel electrophoresis.

To summarize, the chimeric constructs, which were designed in the present study based on 2′-*O*-modified oligoribonucleotides with the modified 3′-terminal internucleotide linkage, possess enhanced stability with respect to 3′-exonucleases and can form stable duplexes with RNA. In complexes with RNA, these chimeric constructs are able to activate RNase H. Hence, they can be considered as potential inhibitors of *MDR 1* gene expression. Specimens of the chimeric oligomers are presently being subjected to tests on cellular systems.

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References

1. *Applied Antisense Oligonucleotide Technology*, Eds. C. A. Stein and A. M. Krieg, Wiley, New York, 1998, 527 pp.
2. A. A. Stavrovskaya, *Biokhimiya*, 2000, **65**, 112 [*Biochemistry* (*Moscow*), 2000 (Engl. Transl.)].
3. D. Novopashina, M. Kuznetsova, and A. Venyaminova, *Nucleosides, Nucleotides, Nucl. Acids*, 2001, **20**, 903.
4. A. S. Boutorine, A. G. Venyaminova, M. N. Repkova, Z. A. Sergeeva, and D. V. Pyshnyi, *Biochimie*, 1994, **76**, 23.
5. C. K. Mirabelli and S. T. Crooke, in *Antisense Research and Applications*, Eds. S. T. Crooke and B. Lebleu, CRC Press, Boca Raton—Ann Arbor—London—Tokyo, 1993, p. 15.
6. S. Shibahara, S. Mukai, T. Nashihara, H. Inoue, E. Ohtsuka, and H. Morisawa, *Nucleic Acids Res.*, 1987, **15**, 4403.
7. K.-H. Altmann, N. M. Dean, D. Fabbro, S. M. Freier, T. Geiger, R. Häner, D. Hüsken, P. Martin, B. P. Monia, M. Müller, Fr. Natt, P. Nicklin, J. Phillips, U. Pieles, H. Sasmor, and H. E. Moser, *Chimia*, 1996, **50**, 168.

8. V. A. Efimov, A. A. Buryakova, S. V. Reverdatto, and O. G. Chakhmakhcheva, *Bioorg. Khim*. 1983, **9**, 1367 [*Sov. J. Bioorg. Chem*., 1983, **9** (Engl. Transl.)].
9. A. G. Venyaminova, A. S. Levina, Z. A. Kosolapova, and M. N. Repkova, *Bioorg. Khim*. 1987, **13**, 1588 [*Sov. J. Bioorg. Chem*., 1987, **13** (Engl. Transl.)].
10. Z. A. Kosolapova, A. G. Venyaminova, and M. N. Repkova, *Bioorg. Khim*. 1990, **16**, 635 [*Sov. J. Bioorg. Chem*., 1990, **16** (Engl. Transl.)].
11. M. A. Kuznetsova, D. V. Pyshnyi, M. N. Repkova, and A. G. Venyaminova, *Bioorg. Khim*., 2000, **26**, 78 [*Russ. J. Bioorg. Chem*., 2000, **26**, 69 (Engl. Transl.)].
12. M. J. Damha and K. K. Ogilvie, in *Protocols for Oligonucleotides and Analogs. Synthesis and Properties*, Ed. E. S. Agrawal, Humana Press, Totowa, 1993, p. 81.
13. A. G. Venyaminova, V. V. Gorn, M. A. Zenkova, N. I. Komarova, and M. N. Repkova, *Bioorg. Khim*. 1990, **16**, 941 [*Sov. J. Bioorg. Chem*., 1990, **16** (Engl. Transl.)].
14. *Molecular Cloning. A Laboratory Manual*, Eds. T. Maniatis, E. E. Fritsch, and J. Sambrook, Cold Spring Harbor Laboratory, New York, 1982.
15. J. F. Ramalho Ortigao, H. Rösch, H. Selter, A. Fröhlich, A. Lorentz, M. Montenarh, and H. Seliger, *Antisense Res. Dev*., 1992, **1**, 129.
16. E. V. Kostenko, R. S. Beabealashvilly, V. V. Vlassov, and M. A. Zenkova, *FEBS Lett*., 2000, **23**, 181.
17. E. Kostenko, M. Dobrikov, D. Pyshnyi, V. Petyuk, N. Komarova, V. Vlassov, and M. A. Zenkova, *Nucleic Acids Res*., 2001, **29**, 3611.

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