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AFFINITY LABELLING OF THE TICK-BORNE ENCEPHALITIS VIRUS RNA REPLICASE PROTEINS BY 4-N-EXO-BASE-SUBSTITUTED PHOTOREACTIVE CTP ANALOGS

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Abstract: 4-N-exo-base-substituted photoreactive analogs of CTP were designed and synthesized. Two flavivirus proteins NS5 and NS3 are shown to be labelled after RNA synthesis in the presence of the analogs, irradiation by UV-light (313 nm) and subsequent [α-^{32}P]NTP incorporation.

Tick-borne encephalitis remains an important public health problem and is caused by flavivirus with single-stranded RNA genome of positive polarity. Genomic RNA encodes 3 structural and 7 nonstructural proteins. The exact structure of flavivirus replicative complex and functions of viral and cellular proteins are not well defined. Flavivirus RNA replication complex is associated with cellular membranes in the perinuclear region (1). The largest tick-borne encephalitis virus (TBEV) protein NS5 (100 kDa) is known to contain a putative methyltransferase domain (2) and GDD motif of viral replicases (3). The purified recombinant NS5 protein possesses RNA-dependent RNA polymerase activity (4). NS3 protein is serine proteinase, RNA-helicase and RNA triphosphatase (5, 6). Involvement of NS1 viral glycoprotein and cellular subunits in viral RNA replication is also possible (7). 5'-end TBEV RNA can bind with two cellular proteins of molecular weights 30 and 22 kDa in the UV RNA-protein cross-linking assay (8) so the host cell proteins might be the putative RNA replicase subunits. All our attempts to isolate active replicative complex by the affinity chromatography on Sepharose CL4B with the immobilized monoclonal antibodies against TBEV NS5 or NS3 proteins were not successful. We could not detect RNA-dependent RNA synthesis in the immunoprecipitates or eluates (9). The development of new methods to study structure and functions of virus proteins in crude enzyme samples is quite desirable. The investigation would be also useful to design new drugs to inhibite virus at the early stage of infection. Recently, affinity modification of TBEV proteins involved in replication initiation by formylphenyl-phosphate analogs of ATP and GTP showed that NS5 protein is responsible for the initiation of minus-strand RNA synthesis at the early stage of infection whereas NS3 protein takes part in the initiation of plus-strand RNA replication at the late stage of infection (10).

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Base-substituted photoreactive analogs of dNTP, containing arylazido groups attached by linkers, were synthesized and characterized (11-15). These analogs have appropriate photochemical properties to permit cross-linking by UV-light 300-360 nm far from own reactivity of nucleic acids and proteins and have a nice elongation substrate properties in reactions catalysed with DNA polymerases (11-13, 15). In this paper base-substituted CTP analogs (**I**)-(**V**): exo-N-[2-(2-nitro-5-azidobenzoylamino)-ethyl]-cytidine-5'-triphosphate (**I**), exo-N-[2-(4-azidotetrafluorobenzoylamino)-ethyl]-cytidine-5'-triphosphate (**II**), exo-N-{2-[O-(4-azidotetrafluorobenzylideneaminooxy)-methylcarbamoyl]-ethyl}-cytidine-5'-triphosphate (**III**), exo-N-[(4-azidotetrafluorobenzylideneaminooxy)-butyloxy]-cytidine-5'-triphosphate (**IV**), exo-N-[(4-azidotetrafluorobenzylidene-hydrazinocarbonyl)-butylcarbamoyl]-cytidine-5'-triphosphate (**V**) (Fig. 1) were synthesized and used for the replication elongation study of TBEV RNA-dependent RNA polymerase.

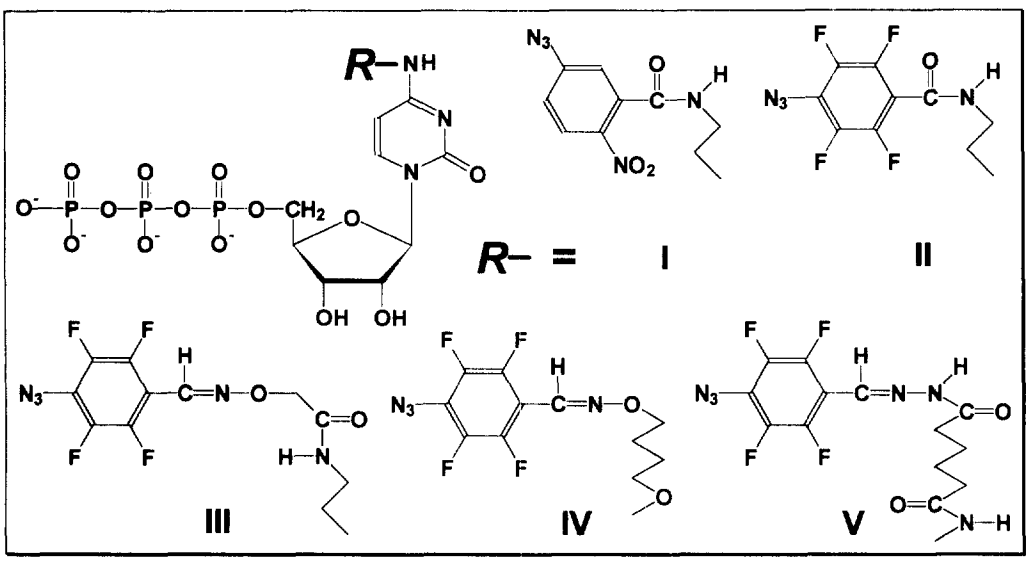

Figure 1. Photoreactive CTP analogs.

Typical experimental procedures.

UV-spectra were recorded on a Specord M40 spectrophotometer (Carl Zeiss Jena, Germany). ^{1}H-NMR spectra were measured on a Bruker-200A instrument. Samples were irradiated with 120 W high-pressure mercury lamp of a KF-4 illuminator (LOMO, St. Petersburg) through glass filters (GS-3, UFS-2) providing band 313 nm, 0.15 mW/cm^2. Photoreactive CTP analogs **I-V** were synthesized and isolated essentially as related deoxynucleotides (15). Spectral, photochemical, substrate properties and HIV-RT photolabelling by dCTP analogs were investigated earlier (11-15).

exo-N-[2-(2-nitro-5-azidobenzoylamino)-ethyl]-cytidine-5'-triphosphate (I).· UV (H_2O), λ_{max} nm (ε, $M^{-1}\cdot cm^{-1}$): 278 (12 000), 322 (8 400). ^{1}H-NMR (D_2O), δ, m.d., (J, Hz): 3.6-3.7 (m, exo N-CH_2-CH_2-N, 4 H);

4.1-4.5 (m, 2 $H_{5'}$, $H_{4'}$, $H_{3'}$, $H_{2'}$, 5 H); 6.0 (d, $J_{1',2'}$ = 4, $H_{1'}$, 1 H); 6.12 (d, $J_{5,6}$ =7.5, H_5, 1 H); 7.11 (d, $J_{o\text{-},p\text{-}}$ =2.5, $H_{o\text{-}}$, 1 H); 7.37 (dd, $J_{p\text{-},m\text{-}}$ =9, $J_{p\text{-},o\text{-}}$ =2.5, $H_{p\text{-}}$, 1 H); 7.9 (d, $J_{6,5}$ =7.5, H_6, 1 H); 8.24 (d, $J_{m\text{-},p\text{-}}$ = 9, $H_{m\text{-}}$, 1 H).

exo-N-[2-(4-azidotetrafluorobenzoylamino)-ethyl]-cytidine-5'-triphosphate (II). UV (H_2O), λ_{max} nm (ε, $M^{-1}\cdot cm^{-1}$): 260 (23 000). ^{1}H-NMR (D_2O), δ, m.d., (*J*, Hz): 3.60-3.75 (m, exo N-CH_2-CH_2-N, 4 H); 4.1-4.5 (m, 2 $H_{5'}$, $H_{4'}$, $H_{3'}$, $H_{2'}$, 5 H); 6.0 (d, $J_{1',2'}$ = 4, $H_{1'}$, 1 H); 6.11 (d, $J_{5,6}$ =7.5, H_5, 1 H); 7.9 (d, $J_{6,5}$ =7.5, H_6, 1 H).

exo-N-{2-[O-(4-azidotetrafluorobenzylideneaminooxy)-methylcarbamoyl]-ethyl}-cytidine-5'-triphosphate (III). UV (H_2O), λ_{max} nm (ε, $M^{-1}\cdot cm^{-1}$): 282 (36 000). ^{1}H-NMR (D_2O), δ, m.d., (*J*, Hz): 3.50-3.65 (m, exo N-CH_2-CH_2-N, 4 H); 4.1-4.5 (m, 2 $H_{5'}$, $H_{4'}$, $H_{3'}$, $H_{2'}$, 5 H); 5.9 (d, $J_{1',2'}$ = 4, $H_{1'}$, 1 H); 6.01 (d, $J_{5,6}$ =7.5, H_5, 1 H); 7.82 (d, $J_{6,5}$ =7.5, H_6, 1 H); 8.43 (s, -N=CH-, 1 H).

exo-N-[(4-azidotetrafluorobenzylideneaminooxy)-butyloxy]-cytidine-5'-triphosphate (IV). UV (H_2O), λ_{max} nm (ε, $M^{-1}\cdot cm^{-1}$): 284 (27 000). ^{1}H-NMR (D_2O), δ, m.d., (*J*, Hz): 1.7-1.9 (m, exo N-C-CH_2-CH_2-C-N, 4 H); 3.50-3.65 (m, exo N-CH_2-C-C-CH_2-N, 4 H); 4.1-4.5 (m, 2 $H_{5'}$, $H_{4'}$, $H_{3'}$, $H_{2'}$, 5 H); 5.87 (d, $J_{1',2'}$ = 4, $H_{1'}$, 1 H); 5.77 (d, $J_{5,6}$ =7.5, H_5, 1 H); 7.82 (d, $J_{6,5}$ =7.5, H_6, 1 H); 8.43 (s, -N=CH-, 1 H).

exo-N-[(4-azidotetrafluorobenzylidenehydrazinocarbonyl)-butylcarbamoyl]-cytidine-5'-triphosphate (V). UV (H_2O), λ_{max} nm (ε, $M^{-1}\cdot cm^{-1}$): 303 (26 000). ^{1}H-NMR (D_2O), δ, (*J*, Hz): 1.7-1.9 (m, exo-N-C-CH_2-CH_2-C-N, 4 H); 2.4-2.6 (m, exo N-CO-CH_2-C-C-CH_2-CO-N, 4 H); 4.1-4.5 (m, 2 $H_{5'}$, $H_{4'}$, $H_{3'}$, $H_{2'}$, 5 H); 6.03 (d, $J_{1',2'}$ = 4, $H_{1'}$, 1 H); 6.2-6.4 (m, H_5, 1 H); 8.1-8.2 (m, H_6, 1 H), 8.29 (s, -N=CH-, 1 H).

Photoaffinity labelling. Porcine embryo kidney (PEK) cells were infected by TBEV (Sofyin strain) at the multiplicity of infection 30 pfu/cell. In 16 or 48 hours after the infection the nuclear fraction were isolated from infected cells according to (10). For affinity labelling the reaction mixtures containing 50 mM Tris-HCl pH 8.0, 5 mM $MgCl_2$, 10 mM KCl, 10 μg/ml actinomycin D and 14 μl nuclear fraction of infected cells were incubated for 5 min at 30^{0}C. Then the different photoreactive analogs of CTP **I-V** were added to the reaction mixtures at the concentrations 10^{-3}-10^{-6} M. After additional incubation for 15 min at 30^{0}C the reaction mixtures were irradiated with UV-light (313 nm) for 5 min. After irradiation [α-^{32}P]ATP was added to reaction mixtures and incubation continued for additional 10 min at 30^{0}C. The exhaustive RNase A hydrolysis continued for 30 min at 37^{0}C.

The proteins were analysed by SDS-PAAG electrophoresis (16), autoradiography and Western immunoblotting with the monoclonal antibodies against TBEV NS3 and NS5 proteins (10, 17). Radioimmunoprecipitation was done according to (9) after 0,1% Triton X100 treatment of reaction mixture with the nuclear fraction from TBEV-infected cells.

Results and discussion.

The affinity labelling by the photoreactive analogs of CTP **I-V** was used to identify the TBEV proteins involved in viral RNA replication elongation. Affinity labelling of the replicative complex at the elongation

stage in crude enzyme samples by original «step-by-step» procedure (11-13) is impossible because of unappropriate composition of samples: low enzyme concentration, competitive host cell DNA-dependent RNA polymerases, complex heterogenous RNA and endogenous NTPs. New labelling procedure consists of several consequent steps (Fig. 2): 1) incorporation of analogs into growing RNA due to RNA polymerase activity, 2) covalent photocross-linking of newly synthesized RNA to proteins through photoreactive arylazido groups; 3) subsequent labelling of RNA-protein complexes by [α-^{32}P]ATP and RNA replicase activity; 4) RNase treatment when described in legends to figures. Last step permits to destruct labelled RNA unprotected by proteins to short oligonucleotides to detect molecular weights of labelled proteins.

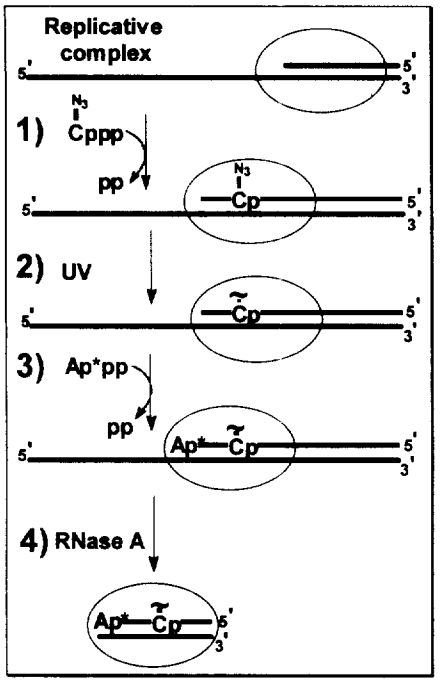

Figure 2. Scheme of affinity labelling of TBEV RNA replicase proteins.

To control the virus specificity and affinity of labelling the following experiments were performed. Host cell RNA synthesis and protein labelling was not observed by the preliminary incubation with Actinomycin D (Fig. 3, lane *3*). Therefore, the whole labelling was resulted from virus-specific or virus-induced activity (Fig. 3, lanes *1-2, 4-6*). DNase I, RNase A and proteinase K treatment showed that labelled products are proteins and RNA.

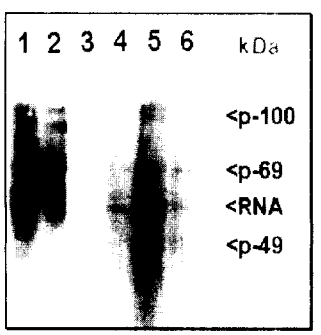

Figure 3. Affinity labelling of TBEV replicase proteins by the analogs **I-V**, incorporated into RNA. Autoradiogram of SDS-PAAG of labelled proteins from the nuclear fraction of uninfected PEK cells (lane *3*) and from the TBEV-infected cells in 48 hs postinfection (lanes *2-5*). Analog concentrations are 1 μM. Analogs: **IV** - lane *1*, **V** - *2*, **III** - *4*, **II** - *5*, **I** - *6* (without RNase A treatment). Protein (p) and RNA positions are indicated on the right.

Radioactive labelling of proteins was observed after incubation neither without CTP analogs with UV irradiation nor with CTP analogs but without UV irradiation (data not shown). It stands for the absence of labelling by NTP-binding, protein kinase activity or other metabolic processes and means that protein labelling occured by photocross-linking through CTP analogs. Optimal concentration for all the analogs appears to be 1 μM (data for analog **IV** in Fig.4, lane *5*) that coincides well with concentration of endogenous NTP in the cellular nuclei (18). The addition of CTP into the reaction mixtures containing analogs **I-V** competitively

decreased TBEV protein labelling and labelled proteins did not appeared in the presence of 1 mM CTP. Among the studied analogs only triphosphate **IV** was toxic for eucaryotic PEK cells in the presence of the compound in the culture medium at the concentrations 10^{-3}-10^{-6} M (data not shown).

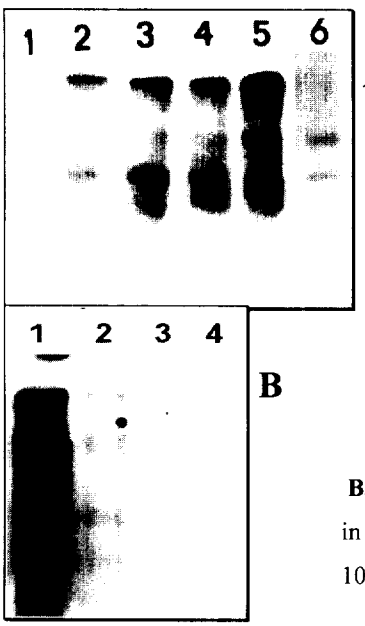

Figure 4. Affinity labelling of TBEV replicase proteins using the reagent **V**. **A.** Autoradiogram of SDS-PAAG of labelled proteins from the nuclear fraction of uninfected PEK cells (lane *1*) and from the TBEV-infected cells in 48 hs postinfection (lanes *2-5*). The analog concentrations: 10^{-3} M - lane *2*, 10^{-4} M - *3*, 10^{-5} M -*4*, 10^{-6} M - *5* (with RNase A treatment). Lane *6* is the immunoblotting of the nuclear fraction from TBEV-infected with the monoclonal antibodies against TBEV NS5 (17) and NS3 (10) proteins.

B. Competitive inhibition of the affinity labelling using the analog **V** by CTP in the concentrations: lane *1* - without exogeneous additional CTP; lane *2* - 10^{-5} M; lane *3* - 10^{-4} M, lane *4* - 10^{-3} M .

The comparison of the autoradiogram (Fig. 4, lanes *1-5*) and Western immunoblotting with monoclonal antibodies against TBEV NS5 and NS3 proteins (Fig. 4, lane *6*) showed the affinity labelled proteins of molecular weights 100 and 69 kDa are TBEV nonstructural proteins NS5 and NS3, respectively. Radioimmunoprecipitation of reaction mixtures with Sepharose CL-4B with immobilized monoclonal antibodies against TBEV NS3 or NS5 proteins or with Protein A-Sepharose after the incubation with monoclonal antibodies against TBEV NS5 and NS3 proteins resulted to radioactive labelled TBEV-specific proteins of molecular weights 100 and 69 kDa complex isolation (data not shown). Currently we can't identify the exact nature of labelled proteins of molecular weights lower than 49 kDa.

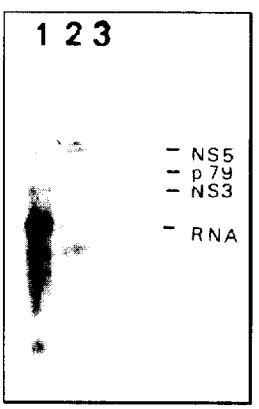

Figure 5. Affinity labelling of TBEV replicase proteins at the different postinfection stages using the analog **V**. Autoradiogram of SDS-PAAG of photoaffinity labelled proteins from TBEV-infected PEK cells in 48 hours P.I. (lane *1*), in 16 hours after infection (lane *2*) and from uninfected control cells (lane *3*) without RNase A reatment.

TBEV new radioactive labelled RNA strands could interact with viral nonstructural proteins NS5 and NS3 in the nuclear fraction of infected cells at the different postinfection stages (Fig.5, lanes *1,2*).

TBEV RNA replication initiation at the early stage of infection was previously shown (10) to occur in the presence of NS5 proteins and single-stranded RNA template. At the late postinfection stage replication initiation is performed by NS3 protein from double-stranded replicative forms RNA (10). Thus, the TBEV RNA replication initiation and elongation differ. In spite of the alternative replication initiation at the early and late stage of infection by NS5 or NS3 protein, respectively, the replication elongation is performed by the same replicative complex consisting of NS5, NS3 and possible NS1 glycoprotein during the whole virus life cycle.

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