Viral Protein Functions Study by Affinity Modification

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Abstract: The knowledge of virus reproduction is necessary to design new safe drugs for inhibition of infections. Ultra-violet irradiation of virus proteins with labeled virus genome fragments permits to identify specific nucleic acid binding proteins. Affinity modification of enzymes with nucleotide derivatives could help to determine NTP-binding proteins and those involved in viral genome replication. Photoreactive analogues of nucleic acids are among the tools used to detect elongation subunits of replicative complexes. Affinity modification approach has already resulted in successful treatment of virus diseases.

INTRODUCTION

Biological complex structure is known to be highly specific and each component can recognize and selectively bind with homologous partners. Affinity modification is the method based on specific interactions between components of biologically active supramolecular complex. Chemical modification resulting in covalent bond formation can fix and thus reveal otherwise undetectable weak interactions and functionally important contact sites between macromolecules and small substrates. The method is also effective to study biopolymer functions. However, the affinity reactions might result not only in specific cross-linking but also in biopolymer damage as well as hidden or unspecific modifications. To detect affinity modification products at least one molecule should be labeled. Usually those are nucleic acids (NA) but in some cases proteins and substrates can be labeled, too.

Affinity modification usually consists of 4 main subsequent steps:

1. isolation or reconstruction of functionally active intermolecular complex;
2. addition of affinity reagent - analogue of the complex component;
3. spontaneous or induced reaction between affinity reagent reactive group and other components of the complex;
4. separation, detection and identification of affinity modification products.

Currently, affinity modification is widely used to study various biological complexes. Several reviews cover the principles of affinity reagent construction, different modification protocols and frequent problems of data interpretation [1-8]. Present work is devoted to virus protein functions study and clinical use of affinity modification approach.

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VIRAL REPLICATIVE COMPLEX STRUCTURES

In order to identify proteins involved in virus reproduction replicative complex can be isolated from infected cells by centrifugation, affinity chromatography with monoclonal antibodies against a replicative complex subunit or with immobilized viral genome fragment. Unfortunately, these obvious approaches often give few or no results due to tight association of virus polymerases with cellular membranes [9-11]. Membranes determine localization of viral replicative complex in the certain cellular compartments, provide optimal orientation and protect biopolymers from protease and nuclease degradation. Moreover, isolation of additional membrane-bound proteins leads to side effects and hides the real replicative complex structure. Affinity chromatography based on binding of immobilized specific antibodies with virus antigen is the most specific among the isolation methods. However, effective elution may require hard conditions (low or high pH, detergents *etc*.) to achieve antigen-antibody complex dissociation. This can result in complex destruction or inactivation [11], making virus membrane-associated replicative complex isolation or reconstruction from recombinant proteins hardly possible [9-11]. Moreover, recombinant proteins may essentially differ from corresponding viral proteins isolated from eukaryotic cells because of specific folding and post-translational modifications [12, 13]. Site-directed mutagenesis of recombinant proteins provides the information about functionally important points of virus proteins and NA.

Other approaches to study virus replicative complex structure are Western immunoblotting of infected cell fractions [14] and radioimmunoprecipitation of [^{35}S]-labeled proteins from infected cells using immobilized antibodies against viral subunits. However, immunoanalysis does not exclude the presence of additional proteins in subcellular fractions. Moreover, immunoglobulines can't penetrate into infected cells, therefore, viral infection inhibition with specific antibodies is hardly possible. One should note that the knowledge of virus replicative complex structure does not mean the understanding of its functions. Development of new methods is necessary to study viral replicative complex functions.

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INTERACTION BETWEEN VIRAL GENOMES AND PROTEINS

Virus replicative complex is known to consist from virus genome, enzyme, new growing NA chains incorporating nucleotide residues. Each component of this complex can modify others and can be modified. According to molecular interaction type affinity modification methods can be aimed at the study of: 1) template-protein binding; 2) interactions between enzyme and nucleotides; 3) binding of polymerase and new synthesized NA; 4) template-primer interaction.

To identify proteins binding with virus genome combination of 3 independent methods including shift mobility assay [15], NA-protein cross-linking under ultra-violet (UV) irradiation and NA-protein blotting [16] could be recommended. All three methods are based on specific binding of studied NA with proteins but experimental conditions differ. Gel retardation of bound with proteins NA in comparison with free genome fragment is performed in native physiological conditions. Shift mobility assay is useful to study the interaction of virus NA with the purified protein(s) but do not allow to identify NA-binding proteins in complex crude extracts.

UV irradiation (290-310 nm) of NA-protein complex is known to result in cross-linking. To dissociate weak unspecific complexes polyanion heparin is used. It is different from the mild conditions of shift mobility assay. Formation of covalent bonds between virus NA and protein permits to reveal natural weak interactions. Since no artificial analogs are added experimental conditions represent *in vivo* ones well enough. The reaction is supposed to occur via a free radical mechanism. Amino acid residues serve as the hydrogen atom donors. The radicals are generated by photoexcitation of NA bases to produce a pyrimidinyl or less likely purinyl radicals along with a radicals on the side chains of amino acid residues. Any nucleotide can take part in the photocross-linking reaction, however, thymine is the most reactive base [4]. All of the 20 amino acids can act as potential cross-linkers. The most reactive amino acids are Cys, Lys, Phe, Thr and Tyr, whereas His, Glu and Asp are moderately reactive.

Photocross-linking has the following disadvantages. Reported yields of photocross-linking varied from 5 to 20%. 100% cross-linking is hardly possible because of photoinactivation of protein, photolysis reactions and photodegradation of cross-linked complex [4]. The optimal excitation wavelength range for all nucleic bases is between 250 and 270 nm. Irradiation is usually carried out at wavelengths shorter than 300 nm, so absorption by other chromophores is possible. This can lead to complex damage and lower yield of specific cross-linking. Single-strand NA breaks can also occur especially when high-intensity irradiation is used [17, 18, 19]. Lowering irradiation intensity up to 10^{18} photons per cm per sec at 260 nm could help to avoid unspecific damage although it could require long irradiation time to achieve a reasonable yield. UV cross-linking can occur simultaneously in many different sites of NA and proteins making site-specific modification of biopolymers by use of their internal photochemical groups impossible. The approach described above has been used to study the role of cellular proteins in the maturation of different viruses [20-25].

The attractive properties of UV cross-linking are non-selectivity and simplicity. The only equipment required is an inexpensive UV lamp (Hg or mineral) or laser. The laser irradiation is more powerfull than UV lamp and can be used for a shorter time.

γ-irradiation could also be used to study NA-protein complexes [17, 18, 19]. Radioprobing is based on the analysis of NA strand breaks produced by ^{125}I, ^{123}I or ^{111}I decay. Isotopes are usually incorporated into duplex or triplex by molecular hybridization with radiolabeled oligonucleotides. γ-irradiation of the isotopes results in emission of a dense cascade of electrons known as Auger electrons, the majority of which are very short-range particles with energies less 1 keV. The short path length of the Auger electrons results in the deposition of a large amount of energy within nanometers from the decay site. Such decay producing NA strand breaks located close to the radioisotope position: 90% of these breaks occur within 10 bp from the decay site. The probability of NA strand breaks decreases with distance from the decay site. Consequently, the distribution of NA breaks is sensitive to conformation changes, in particular, inside complexes with proteins [18, 19]. Unfortunately, γ-irradiation is dangerous and should be used in the absence of other available techniques only.

Blotting is widely used method for direct detection of viral NA-binding proteins [16]. After electrophoresis denaturated proteins are transferred to the nitrocellulose or nylon membranes and then are renaturated in the presence of 8% glycerol and Mg^{2+}. The separation of complex mixtures permits to study the interaction between proteins and viral genome without preliminary time and money consuming protein purification steps. Unfortunately, hard conditions of NA-protein blotting including heat denaturation of protein samples before electrophoresis might damage NA-binding capacity and make proteins functionally inactive. Consequently, the lack of signals in NA-protein blotting does not necessary mean the absence of specific NA-binding proteins in the studied mixture.

There are several kinds of natural modifications of cellular and viral biopolymers. Posttranslational modifications such as phosphorylation, glycosylation and fatty acid attachment are known to modulate the activities of many enzymes in eukaryotic cells [26]. Nucleotide residues of tRNA can also be modified. However, the natural changes of biopolymer chemical structure do not result in production of chemically reactive groups and can't be directly used for the affinity modification. Exogenous chemically active groups have to be introduced for production of covalent linkages between viral NA and surrounding proteins. Formaldehyde is usual choice to produce covalent binding of ε-NH_2 groups of Lys residues with amino- or imino- groups of NA [17]. Glutaraldehyde and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimid react with primary amino groups of proteins and NA yielding Schiff base. Unfortunately, acid activation of NA that might provide better chemical modification of binding proteins is not acceptable to study NA-protein complexes because of low pH and high ionic strengths. Preliminary alkylation of purine nucleotide residues using dimethylsulfate with subsequent heating at neutral pH also result in bonds formation between NA and

amino groups of proteins [17]. When Mg^{2+} ions in the catalytic center of enzyme (RNA polymerase) had been replaced with Fe^{2+}, generated hydroxyl radicals cleaved both template and protein subunits [27]. This approach could be applied to locate the interaction sites between viral genome and enzyme. Multiple chemical modifications of nucleotides in that case result in rearrangements of compex structure but provide higher yield of cross-linking products than UV irradiation. Unfortunately, the chemical modification conditions are too hard to study viral complexes in living cells and that approach can't be applied to inhibit infections.

To reveal consensus NA region binding with an individual virus protein or biological complex the selection method of NA ligands from *in vitro* synthesized, randomized pools, called SELEX has been proposed [28]. Consensus RNA ligand that binds and specifically inhibit human immunodeficiency virus 1 (HIV-1) reverse transcriptase has been identified using this procedure [29]. To map NA-protein interaction sites chemical and enzymatic footprinting can be used. Both methods are based on modification or cleavage of NA unprotected by proteins using dimethylsulfate (DMS), metal-EDTA-generated hydroxyl radicals or DNase I, respectively [30].

AUTOCATALYTIC AFFINITY MODIFICATION FOR IDENTIFICATION OF POLYMERASE INITIATION SUBUNITS

Polymerases (nucleotidyltransferase) are the enzymes responsible for template-dependent NA synthesis from NTP or dNTP. All known polymerases catalyze the same chemical reaction - the formation of phosphodiester bond between 3'-OH end of a new polynucleotide chain and α-phosphate group of (d)NTP. According to chemical nature of template and newly synthesized NA strand polymerases could be subdivided into four classes: DNA-dependent DNA polymerase, DNA-dependent RNA polymerase, RNA-dependent DNA polymerase and RNA-dependent RNA polymerase. All four classes are represented among real virus-specific polymerases. Polymerase active centers share common structure and properties [31]. Affinity modification of the enzymes with NTP analogues in the presence of specific templates is one of the most informative approaches since template NA plays the central role in the correct complex formation and nucleotide can be easily modified. Numerous substances containing ribonucleoside residues with free 2',3'-glycol group such as dinucleoside-monophosphates, nucleoside-5'-di- and monophosphates, nicotinamidadenindinucleotide and flavinadenindinucleotide can serve as initiating substrates for different polymerases. Different chemical modifications of nucleotides can be utilized in cross-linking approach (Table **1**) [32-41]. NTP-binding virus proteins were easily identified with radioactive, biotin-labeled nucleotides [42] and its analogues (Table **1**). However, a variety of phosphoproteins without enzymatic activity might be labeled this way [43]. Therefore, this approach reveals NTP-binding proteins but does not prove the target protein role in template-dependent synthesis.

The most promising method to study virus reproduction mechanisms is autocatalytic affinity labeling [44, 45]. The method has been originally proposed to study transcription initiation including binding of enzyme with initiating substrate and the first phosphodiester bond formation. This approach is based on the covalent modification of polymerase active centers with derivatives of initiating

Table 1. Affinity Modification of Virus Proteins

Reactive group	Affinity group	Target (*)	Labeling	Identified product	References
Aldehyde	Pyridoxal-5'-Phosphate	Influenza virus transcriptase	$NaB[^3H]_4$	core protein PB1	[32]
Aldehyde	Pyridoxal-5'-Phosphate	HIV reverse transcriptase	$NaB[^3H]_4$	P66 subunit (Lys-263)	[33]
Aldehyde	$NaJO_4$ oxidized [α-^{32}P]ATP	Simian virus 40 and polyoma virus nucleoprotein complexes (infected cell extracts)	[α-^{32}P]ATP	T antigens	[34, 35]
Aldehyde	o- and p- formylphenyl esters of AMP, ADP, ATP GMP, GDP and GTP.	Tick-borne encephalitis virus replicative complex (infected cell extract)	[α-^{32}P]-labeled second NTP	NS3 (Lys-1800 and/or Lys-1803) and NS5 proteins	[36, 37]
Aldehyde	$NaIO_4$ - oxidized $tRNA^{Trp}$	Avian myeloblastosis virus reverse transcriptase	$NaB[^3H]_3CN$	α subunit >> β subunit	[38]
1. Aldehyde 2. 2-cloro-ethylamino-group	site-specifically tiolated oligo(dT)	HIV-1 reverse transcriptase with polyA template	elongation with [α-^{32}P]dTTP after cross-linking	P66 and P51 subunits P66 subunit	[39]
Trans-diamine-dichloro-platinum	$tRNA_3^{Lys}$	HIV-1 reverse transcriptase (nucleocapsid)	1. 5'-[^{32}P]-$tRNA_3^{Lys}$ 2. 5'-[^{32}P]-oligonu-cleotides	P66 and P51 subunits	[40]
N-hydroxy-succinimide ester	RNA duplex with 4-thiolated UMP-23.	HIV-1 Tat protein	Tat protein with C^{13} and N^{15} Arg residue for mass spectrometric analysis	Tat protein (Lys-51)	[41]

nucleoside-5'-triphosphate (NTP) and subsequent elongation with radioactive labeled NTP. Substrate analogues bound outside the active center or attached to other proteins can't be elongated and remain unlabeled. Thus, autocatalytic affinity labeling provides a highly specific introduction of the radioactive label near substrate binding centers of enzymes. The whole process of autocatalytic affinity modification can be illustrated with the following scheme:

$$E\text{-}NH_2 + OHC\text{-}Rp_nN_1 \xrightarrow{NaBH_4} E\text{-}N{=}CH\text{-}Rp_nN_1 \longrightarrow$$

$$E\text{-}NH\text{-}CH_2\text{-}Rp_nN_1 + pp^*pN_2 \longrightarrow E\text{-}NH\text{-}CH_2\text{-}Rp_nN_1{}^*pN_2$$

where $E\text{-}NH_2$ is the enzyme containing lysine residues, $OHC\text{-}Rp_nN_1$ is aldehyde-containing nucleotide derivative, n equals 1, 2 or 3 and pp^*pN_2 is the radioactive labeled second NTP in the sequence complementary to template.

Nucleotide analogues with aldehyde attached to phosphate group can react with nucleophilic amino groups of the protein. Aldehyde-histidine adducts are stable enough to survive electrophoresis, in contrast with the lysine-aldehyde derivatives, which are unstable unless stabilized by reduction [36, 44, 45]. Primary NH_2-groups such as ε-amino groups of Lys residues or α-amino group of the N-terminal amino acid of protein form Schiff bases with aldehydes of nucleotide derivatives. This reaction is reversible. To increase the stability of link between affinity reagent and protein Schiff bases must be reduced with $NaBH_4$. Subsequent addition of [α-^{32}P]NTP results in attached dinucleotide synthesis which is catalyzed with modified polymerase itself. Thus autocatalitic affinity labeling of purified or crude polymerase can be achieved.

Labeled sites nearby the active center might be localized by several cleavage at specific amino acid residues. Hydroxylamine is known to split the amide bond between the neighboring Asn and Gly residues of polypeptide at pH 10.0 and CNBr cleaves after Met residues. Analysis of radioactive single-hit and exhaustive cleavage products provides information for the label position mapping. Comparison of peptides produced by a single-hit hydroxylamine cleavage with limited and exhaustive CNBr cleavage products allowed us to map TBEV NS3 protein Lys1800 and/or Lys 1803 residues as the attachment sites for affinity analogues of the initiating substrate [36].

DETERMINATION OF VIRAL POLYMERASE ELONGATION SUBUNITS

Chemical or enzymatic introduction of nucleotide analogues in NA can result in higher level of their cross-links with surrounding proteins than after natural NA-protein cross-linking under UV irradiation. Photon flux required to obtain cross-linking is greatly reduced and protein photodenaturation is minimized. The photomodification with modified NA occurs in the millisecond range and permits to study dynamic events [8].

Different photoreactive groups with various length and rigidity of spacers connecting the photoreactive groups with heterocycles were used for photocross-linking (Table **2**) [46-63]. The 5-base-substituted pyrimidine analogs and 8-base-substituted purine analogues are widely used. Chemical groups attached at these positions of nucleotides are exposed into the major groove of DNA double helix so they do not significantly alter NA structures and binding with polymerases. After mild irradiation cross-linking yield obtained could reach 60% [17]. Irradiation at wavelengths greater than 300 nm diminishes the risk of photodamage for NA and proteins. At 308 nm 5-bromouracil cross-links to the aromatic amino acids Tyr, Trp and His, as well as to Cys. Arylazido nucleotides have appropriate photochemical properties to permit cross-linking by UV-light irradiation with wavelength more than 320 nm, i.e. out of photosensitivity range for NA and proteins. Irradiation with UV or visible light of arylazido nucleotide analogues results in dissociation of $RN\text{-}N_2$ bond with generation of molecular nitrogen and singlet nitren [8]. Singlet aromatic nitren is highly reactive and participates in intra- and intermolecular reactions. Presence of reduction reagents such as β-mercaptoethanol or dithiothreitol in reaction mixtures prevents photocross-linking. The main part of arylazido nucleotides reacts before contact with NA and proteins. Among azidonucleotides the best elongation substrates for many known polymerases are 5-C-base-substituted pyrimidines. N-exo-base-substituted CTP and ATP analogues are the worse substrates for enzymes of template synthesis. The available data concerning modification points of proteins labeled with arylazide nucleotides are insufficient to draw certain conclusions. However, photoadducts are known to be predominantly formed with nucleophilic groups, mainly with Lys, Tyr and Trp residues. Preirradiation of $8\text{-}N_3$-pAp retained its ability to modify the enzyme in the dark at the same point as under irradiation of the mixture of the enzyme with the photoreagent [8].

Photoreactive groups can be also attached to proteins. For example, enzymatic protein phosphorylation with [^{35}S]ATPγS and subsequent alkylation converts protein-thiophosphate to photoreactive protein [8]. The absence of photocross-linking in that case could be caused not only by the photoreaction failure but also by the absence of direct contacts between a photoreactive group and target. Therefore, photoaffinity modification data should be considered with some caution. One should note that high yield of photoaffinity modification is due to multiple cross-linking between different nucleotide and amino acid residues. Consequently, exact localization of polymerase active center near certain amino acid residues is hardly possible by means of this method.

Photoaffinity labeling experiments can be performed in two different ways: 1) template synthesis of NA in the presence of substrates containing photoreagent and at least one radioactive nucleotide with subsequent UV irradiation; 2) autocatalytic affinity labeling including preliminary irradiation of template-primer-enzyme complex with subsequent addition of labeled nucleotide [39]. Whereas experiments of type 1 lead to multiple labeled products affinity labeling of type 2 is more specific. Comparison of autoradiograms and immunoblotting with monoclonal antibodies against viral proteins as well as radioimmuno-precipitation data could result in identification of affinity labeled products (Table **2**).

Photoaffinity modification of virus proteins *in vivo* is complicated by problems of irradiation and analogues

Table 2. Photoaffinity Modification of Virus Proteins

Reactive group	Affinity group	Target	Labeling	Detected modification products	References
Thd	1. dTTP 2. $(rA)_{12-18}$*$(dT)_{10}$	HIV-1 reverse transcriptase	1. [α-^{32}P]-dTTP 2. 5'-[^{32}P]$(rA)_{12-18}$	1. P66 and P51 subunits 2. P66 subunit	[46]
Thd	dTTP	HIV-1 reverse transcriptase	[*methyl*-^{3}H]-PAR	P66 subunit (Lys-73)	[47]
1. 4-thio-Urd 2. Thd 3. 5-azido-dUrd	Primers with 1. 4-thio-UTP 2. Az-dTTP 3. T505 4. 5-azido-dUTP	HIV-1 reverse transcriptase	[α-^{32}P]-dCTP during *in situ* primer elongation	P66 subunit	[48]
4-thio-Urd	$tRNA_3^{Lys}$ with 4-thio-UMP	HIV-1 reverse transcriptase	5'-[^{32}P]- position to 4-thio-Urd	P66 subunit (-1- and 16-thio-$tRNA_3^{Lys}$) P66 and P51 subunit (3:1, 36-thio-$tRNA_3^{Lys}$)	[49]
4-thio-Urd	RNA duplex with 4-thio-UMP	HIV-1 Tat protein	Tat protein with C^{13} and N^{15} Arg residue for mass spectrometric analysis	Tat protein (Arg-55)	[41]
5-Br-Urd	RNA with 5-Br-UMP	bacteriophage R17 coat protein	[α-^{32}P]NTP	20-50% cross-links, little RNA and significant protein photodamage	[50]
5-Br-Urd (laser/308 nm) 5-I-Urd (laser/325 nm)	RNA hairpin	bacteriophage R17 coat protein	[α-^{32}P]-NTP	~ 40% of cross-links using 5-Br-Urd > 90% of cross-links using 5-I-Urd	[51]
5-I-Urd	randomized RNA library with 5-Br-UMP	HIV-1 Rev protein	[α-^{32}P]-NTP	Specific RNA-ligand for HIV-1 Rev protein	[52]
5-I-Cyd	RNA hairpin	bacteriophage MS2 coat protein	[α-^{32}P]-NTP introduction by transcription	20% of cross-links 40% of unreacted RNA 40% of RNA cleavage products	[53]
5-azido-2-nitro-benzoyl-amino group	primer, containing 5-C-substituted dUMP at different positions	bacteriophage T4 DNA polymerase (44, 62, 45 accessory and 32 ssDNA-binding proteins, template)	5'-[^{32}P]-primer	T4 DNA polymerase	[54]
4-azido-phenacyl	primer, containing 5-C-substituted CMP at different positions	bacteriophage T4 RNA polymerase	5'-[^{32}P]-primer	> 50% cross-links to RNA polymerase	[55]
azidoaryl group	Dipyrido-diazepinone BI-RG-587	HIV-1 reverse transcriptase	[^{3}H]-labeled PAR	P66 subunit (Tyr-181 and Tyr-188)	[56, 57]
4-azido-2,3,5,6-tetrafluoro-benzoyl-amino group	4-N-exo- and 5-C-substituted dCTP and primers	HIV-1 reverse transcriptase	5'-[^{32}P]-primers [α-^{32}P]-dNTP after irradiation	P66 >> P51 subunits P66 subunit	[58, 59]
4-azido-2,3,5,6-tetraftluoro-benzyliden group	4-N-exo-substituted CTP	Tick-borne encephalitis virus replicative complex (infected cell extracts)	[α-^{32}P]NTP after irradiation	NS3 and NS5 proteins	[60, 61]
4-azido-2,3,5,6-benzoyl-amino and 4-azido-anilin groups	5-C-substituted UTP	Tick-borne encephalitis virus replicative complex (infected cell extracts)	[α-^{32}P]NTP after irradiation	NS3 and NS5 proteins	[61]
(3,3,3-tri-fluoro-methyl-3H-dazirin-3-yl)styryl group	5-C-substituted ddUTP	HIV-1 reverse transcriptase with oligo(dT)-polyA)	[γ-^{32}P]-PAR	P66 subunit (3%)	[62]
(3,3,3-tri-fluoro-methyl-3H-dazirin-3-yl)styryl group	5-C-substituted ddUTP	HIV-1 reverse transcriptase with oligo(dT)-polyA	[γ-^{32}P]-PAR 5'-[^{32}P]-$(dT)_{16}$	2. P66 subunit (~ 15-20% cross-linking in 2-d labelling variant)	[63]

delivery. According to our unpublished data less than 30% photoreactive nucleotide could penetrate into living eukaryotic cells. Among the studied nucleotide derivatives only exo-N-[(4-azidotetrafluorobenzylideneaminooxy)-butyloxy]-cytidine-5'-triphosphate was toxic for eukaryotic cells at the concentrations 10^{-3}-10^{-6} M [60].

Inhibition of virus reproduction in infected cells could be achieved by treatment with derivatives of oligonucleotides complementary to virus genome [64, 65]. Oligonucleotide transport into cells is known to be more efficient in comparison with NTP analogues due to specific receptors for NA exhibited on the cellular surface [66]. Moreover, "antisense" oligonucleotides bring additional target specificity. Inhibition of HIV-1 transcription in infected cells by oligonucleotides corresponding to transcription initiation site and nuclear factor Sp1 binding site has been shown [67]. Unfortunately, oligodeoxyribonucleotides get destroyed with cellular enzymes within several hours after administration [66]. Specific delivery of modified nucleotides in infected cells using immunoliposomes with monoclonal antibodies against virus-specific proteins might locally inhibit virus infection [68].

VIRUS INACTIVATION IN CLINICAL TRIALS

Successful experiments in drugs development and inhibition of virus reproduction have already led to clinical trials. Among currently available chemotherapeutic agents the most widely used nucleoside analogues are 3'-azido-2',3'-dideoxythymidine (AZT), 2',3'-dideoxyinosine (ddI) and 2',3'-dideoxycytidine (ddC) which terminate viral NA synthesis after phosphorylation. Unfortunately, their unspecific action results in toxicity for host cells which strongly limits their effectiveness [69]. At present, there are nonnucleoside inhibitors of viral enzymes. For example, the dipyridodiazepinone (nevirapine or BI-RG-587) inhibits HIV-1 reverse transcriptase in a noncompetitive manner with respect to template, primer, and nucleoside triphosphates [70]. The dipyridodiazepinone displays a higher specificity for HIV-1 reverse transcriptase in comparison with other human DNA polymerases as well as feline and simian virus reverse transcriptase and even HIV-2 [69]. An azido photoaffinity analogue of nevirapine BI-RJ-70 has been used to localize the specific binding sites of BI-RG-587 with tyrosine residues of p66 subunit of HIV-1 heterodimeric reverse transcriptase [70]. A series of thiobenzimidazolones are also nonnucleoside inhibitors of HIV-1 reverse transcriptase but are inactive against HIV-2 [69]. In spite of great attempts in the field the finding of a new inhibitor of virus reproduction still remains "the state of art" work.

For elimination of viral contamination in donor blood a number of different methods based on virus removal and/or inactivation were explored. Removal can be achieved by washing, filtration or adsorbtion. Inactivation methods include heat, intrinsically active chemical agents such as hydrolyzable diol epoxides, ozone, halogenated oxidizing agents, photo- and γ-irradiation without chemical addition, and photo-irradiation in the presence of sensitizing agents that are inactive in the dark [71]. Among these approaches photo-irradiation is the most specific method because the sensitizer can be concentrated on the virus particles before activation. The sensitizing agent must have a high binding capacity and interact specifically with viral envelope proteins or lipids. Inactivation may occur because photoinduced damage renders the virus unable either to enter a prospective host cell or to replicate because of defective genome. Cationic organic dye methylene blue and its derivatives thionine and thiopyronine are well known "virucidal" agents of that type [71, 72]. A photochemical virus inactivation in blood plasma using methylene blue has been successfully introduced in Europe since 1992 [73]. The targets for methylene blue are viral capsids or lipid envelopes for one group of viruses or NA for other. Singlet oxygen and 8-oxo-7,8-dihydro-2'-deoxyguanosione are generated by the photosensibilization. Under deoxygenated conditions single-strand NA breaks have been also observed suggesting the existence of an oxygen-independent mechanism of DNA cleavage, too. Efficacy of virus inactivation depends on the reagent concentration, irradiation conditions and the treated virus. Unfortunately, intracellular viruses are not inactivated by methylene blue photosensibilization. Up to 20% blood cells remains infected [73].

Extracorporeal photochemotherapy, also known as photopheresis, is an immunomodulatory therapy including oral administration of the photoreactive drug 8-methoxypsoralen followed by *ex vivo* UV irradiation of circulating lymphocytes. It has been approved for cutaneous T cell lymphoma treatment and recommended for management of autoimmune disease, graft rejection and HIV infection [74]. Lymphocytes have been shown to be the cell population most affected by HIV and photopheresis. Moreover, conformational changes of viral antigens after photochemotherapy induce a better specific cytotoxic immune response than native antigens resulting in lysis of virus-producing T-cells by cytolytic T lymphocytes.

Photodynamic therapy is based on the excitation of a systemically administered photosensitizer with a light of a wavelength corresponding to an absorption peak of the photosensitizer. Photoreactions occur when a chromophore is exposed to light and is excited from the electronic ground state to a high energy singlet or triplet states. Sensitizer in excited triplet state in turn could provide the singlet oxygen which can damage lipids, proteins or other biopolymers. For example, halogeno derivatives of fluorescein are efficient producers of singlet oxygen [75].

Hydrophobic sensitizers bear no charged groups and thus have negligible solubility in water or alcohol (phthalocyanines and related naphthalocyanines). Hydrophilic sensitizers have three or more charged side groups and are soluble in water at physiological pH (sulfonated aluminium phthalocyanine and the tetra- and trisulfonates; sulfonated Zn, Fe(II) or Ga phthalocyanines; chlorin and pheophorbide chelated with vanadyl chloride; benzoporphyrin derivative conjugated with polyvinyl alcohol). Amphiphilic sensitizers have one or two charged substituents and are somewhat soluble in alcohol or water at physiological pH (porphyrin-based reagents; meta-

tetrahydroxyphenylchlorin; lower sulfonated phthalocyanines; pheophorbide a; bacteriochlorine a; ketochlorin) [76].

A number of photodynamic perylenequinone pigments have been isolated from fungi and other microorganisms. One of them - hypocrellin A isolated from the Chinese medicinal fungus *Hypocrella bambuase* inactivates HIV when irradiated with light. Antiviral activity of these compounds is caused their lipophilicity, virus-binding affinity and singlet oxygen action. Perylenequinones are not cytotoxic and have relatively little antiviral effect without irradiation [77]. In spite of different original intracellular localization of photosensitizers (lysosomes, perinuclear region or diffusely throughout the cells) irradiation results in relocalization because of photodynamic permeabilization of the lysosome membranes allowing small molecules to leak out [76].

More hydrophilic photosensitisers bind preferentially to albumin and reach tumours via their increased vascular permeability. A combination of "leaky" tumour vasculature and reduced lymphatic drainage can encourage the accumulation of sensitizer aggregates or complexes with proteins in the interstitial space. Tumour cells with increased capabilities for phagocytosis or pinocytosis can take up such complexes [78]. The tendency of the photosensitiser to be taken up to a greater extent by hyperproliferative cell populations in comparison with the resting cells and the characteristic accelerated uptake by neovascular endothelial cells provide the selectivity of photodynamic therapy of cancer. There may be three separate mechanisms for tumour distruction in photodynamic therapy: 1) direct damage to tumour cells; 2) damage to the endothelial cells of the tumour microvasculature; 3) macrophage-mediated immune infiltration of the tumour [78]. Tumour cells in isolation are not able to accumulate higher level of the chemical compounds than are normal cells. Retention of porphyrines in malignant tissue could be explaned by the following reasons. All the rapidly proliferating cells including cancer need more cholesterol for membrane biosynthesis. It results in upregulation of the LDL receptor gene expression. Lipoproteins are known to be the major carrier of lipophilic porphirins in the bloodstream and therefore, may be a way of these compounds entry into cells. The ability to target photosensitizers to macrophages may have several applications. Laser-induced fluorescence imaging can be used for the diagnosis of early cancers and fatty-streaks stage of atheroma. However, in the case of LTD conjugates with conventional cytotoxic drugs, a highly specific uptake by tumour-associated macrophages could reduce the ability of macrophages to keep the tumour in check. Other severe problems are instability of such conjugates and complexes of various drugs with LDL and their changed affinity towards rapidly dividing cells [78]. Photosensitisers may accumulate in tumours, atherosclerotic plaques, areas of inflammation, healing wounds and other pathological lesions by virtue of macrophage receptors. In addition to the complement-opsonized phagocytosis of liposomes by macrophages phospholipids on the surface of liposome are recognized by macrophage receptors. Injected hydrophobic sensitizer forms compexes with circulating LDL. As a result, the LDL may become oxidised, have a new conformation or remain uncharged. Modified LDL are delivered to lysosomes mostly via the scavenger receptor of macrophages whereas normal LDL enter via the conventional apoB/E receptor of macrophages.

Approval of the first photosensitizer, Photofrin (porfimer sodium, the complex mixture of crude sulfonated phthalocyanines [74]) by health boards in United States, Canada, Japan and the Netherlands for use against certain types of solid tumors illustrates the progress of photodynamic therapy from a laboratory research to clinical reality. Hematoporphyrin derivatives are currently clinically applied, too. It is local and superficial therapy because light has a limited penetration inside tissues. The majority of preclinical and clinical photodynamic therapy studies have used red light with maximum penetration depth of approximately 1 cm. Unwanted side effects from deep penetration of red light can be eliminated with the use of green light (514 nm) with hematoporphyrin derivatives. In this case tissue necrosis occurs up to a depth of 3.3 mm [78]. Red-light therapy is limited by intestinal toxicity. Therefore, less penetrating green light with higher doses can be used in the peritoneal cavity.

Photodynamic therapy resulting in abnormal tissue ablation is desired treatment of many diseases including atherosclerosis, restenosis, rheumatoid arthritis, age-related macular degeneration and closure of leaking vessels without damage of normal vessels. Moreover, photodynamic therapy can result in modulation of immunological behavior and transient suppression of immune responsiveness.

Illumination of small animals with red light without skin photosensitivity leads to previously injected photosensitizer activation in the circulation system. This procedure termed as transcutaneous photodynamic therapy might be the novel approach to treat for autoimmune diseases, to abrogate the development of an acute adjuvant enhanced arthritis and contact hypersensitivity as well as to prolong the survival of skin allografts. Sublethal doses of irradiation can stimuli interleukin-1 and tumor necrosis factor - α production [80].

Recent achievements in virus replication studies has already led to the development of virus inactivation approaches for the clinical use. Further attempts should be aimed to increase both specificity and efficiency of affinity modification of virus replicative complexes inside infected cells. This goal could be achieved using both specific targeting of chemical reagents into infected cells or antisense oligonucleotide or RNA derivatives.

ABBREVIATIONS

NA = Nucleic acids

UV = Ultra-violet light

HIV-1 = Human Immunodeficiency Virus of type 1

HIV-2 = Human Immunodeficiency Virus of type 2

TBEV = Tick-borne encephalitis virus

LDL = Low-density lipoproteins

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