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PLENARY LECTURES

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Newly synthesized 2-styryl-8-hydroxyquinolines (SQS) and their derivatives: inhibition of human immunodeficiency virus-1 (HIV-1) replication in cell culture

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Introduction During the last years, efforts against the HIV/AIDS pandemic were directed to find substances that block viral replication and slow down the progress of infection. The life cycle of HIV is comprised of different steps that are adequate targets for chemotherapeutical interventions. Nucleoside and non-nucleoside reverse transcriptase inhibitors (NRTIS, NNRTIS), protease inhibitors (PIs) and entry inhibitors are components of routine treatment regimens (4). A very important step of HIV replication is the integration of proviral DNA into cellular genome so ensuring a stable maintenance of the viral genome and persistence of the virus in the host. Integrase (IN), the enzyme responsible for integration and coded for by *pol* gene is therefore an attractive target for novel drugs because of its central role in the life cycle of HIV.

A number of IN inhibitors have been characterized *in vitro* – synthetic (Diketo acids- DKA (L-708,906), Diketo aryls (5CITEP), Pyranodipyrimidines, Styrilquinolines - SQ, etc.), and natural (L-Chicoric acid, Coumarins, Cucurmin, etc.) compounds. They can be divided into two groups: those that inhibit the 3'-processing reaction and those that preferentially inhibit the strand transfer reactions. SQ – novel potent integrase inhibitors, inhibit the 3' processing. Studies showed that the subdivision of IN inhibitors into two distinct groups was not so clear (5, 6). In 2007 the first integrase inhibitor – Raltegravir (Isentress^{TM}) (a strand transfer blocker) was licensed after successful clinical trials (7).

Table 1 Newly synthesized SQS

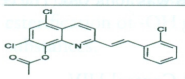
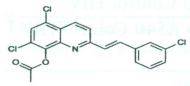
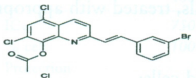
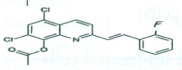
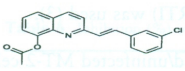
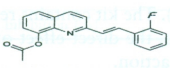
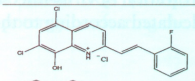
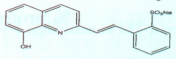
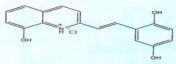
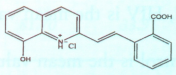
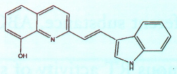
Formula	Code No	Molecular mass (M)
	100	392.7
	101	392.7
	103	437.1
	105	376.2
	201	323.8
	205	307.3

Table 2 Derivatives of the initial compounds

Formula	Code No	Molecular mass (M)
	105H Derivative of 105	370.63
	301S Derivative of 205	349.34
	302H Derivative of 205	315.75
	303H Derivative of 205	327.76
	304B Derivative of 205	302.37

SQs are synthetic compounds like DKAs, designed (8) to chelate the divalent (Mg^{2+} in the integrase core domain. The SQs competitively inhibit the 3'-processing with higher affinity than the strand transfer inhibitors. The reason for this is their 5–10 times higher affinity to the donor than to the

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acceptor site of IN. Once the DNA-IN complex is built, the SQs cannot inhibit the 3'-processing because they cannot destroy the complex integrity (2, 8). Essential for the inhibition activity are 7-COOH and 8-OH groups probably participating in the chelation reaction. In current paper, data are presented on inhibition of HIV-1 replication in cell culture by six newly synthesized 2-styryl-8-hydroxyquinolinyl acetates (designated as initial substances) (Table 1) and five derivatives of them – salts, bases and hydrochlorides, synthesized after evaluation of anti-HIV activity of the initial substances (Table 2).

Materials and methods The chemical synthesis of all SQs was performed in the Veterinary Faculty of Trakia University, Stara Zagora, Bulgaria by A. Pavlov and S. Chervenkov.

MT-2 cells were used as a classical model for acute infection with HIV-1 (X 4 strain) and testing for inhibitory effect (9). Cells were grown in RPMI medium with 10% FCS.

$HeLaP_4$ monolayer epithelial cells, engineered to produce CD4 receptors, a kind gift from Prof. J.-L Darlix - Ecole Normale Superieure – Lyon, France, were grown in DMEM supplemented with 10% FCS.

As a source of HIV-1, the supernatant of H9/HTLV III B cell line was used. The supernatants were stocked with known p24 antigen content, RT activity and infectivity.

The following parameters were studied: cytotoxic concentration 50 – CC50 (preventing death of 50% on MT-2/$HeLaP_4$ cells), maximal nontoxic concentration – MNC (the highest concentration causing no cytotoxicity on both cell lines), and inhibitory concentration 50 – IC_{50} (concentration inhibiting by 50% the viral replication). CC50 and MNC were detected by MTT – uptake assay (9). IC_{50} was studied only on MT-2 cells by micro titer infection assay exploring the cytopathic effect of HIV, using MTT test (9). Experiments under conditions of acute infection were performed in 96-well microplates with 6–8 parallels/experiment. Cell controls (MT-2 cells with medium only) and viral controls (virus infected MT-2 cells without treatment with the substances tested) were run with every experiment. For anti-virus assay 50 µl HIV (undiluted or diluted to obtain multiplicity of infection about 0.1) was added to each well except the cell controls. Virus was allowed to attach the cells for an hour at 37 °C/5% CO_2. All the compounds were prepared in 10 × dilutions (one dilution/column of plate). The plates were incubated for 72 hours at 37 °C/5% CO_2. After that, MTT test was performed as described (9) and absorbance of viable cells was measured calorimetrically at A540 nm. For all experiments, the mean value of each column was calculated. The 50% cytotoxic concentration (CC_{50}) of the test compound was defined as the concentration reducing the absorbance (A540) of mock-infected cells to 50% compared to the cell controls. IC_{50} was expressed as the concentration where 50% protection of virus-infected and substance treated cells was achieved. The cell survival (% protection) was calculated according to the following formula:

$$\%\ \text{protection} = \frac{\text{A540X} - \text{A540 Control HIV}}{\text{A540 Cell Control} - \text{A540 Control HIV}} \times 100,$$

Where, X is the mean value of A540 of HIV-infected cells, treated with appropriate concentration of the substance studied;

Control, HIV is the mean value of A540 of HIV-infected cells;

Cell control is the mean value of A540 of un-infected and un-treated cells.

As a referent substance, ABC (Abacavir - well known NRTI) was used (3).

Endogenous RT activity of supernatants of HIV-1 infected/uninfected MT-2 cells treated/untreated with SQLs was tested by HS-Lenti Kit-RT assay (Cavidi, Sweden). The kit contains recombinant RT (rRT) as a standard, which makes possible RT quantitation (11). Also, the direct effect of the compounds on rRT activity was measured to prove RT as a target of antiviral action.

Results and discussion Table 3 shows CC_{50} and MNC of newly synthesized SQs measured through MTT-test in MT-2 and $HeLaP_4$ cell lines. In MT-2 cells, CC_{50} values were so close to MNC that

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practically could not be detected. Results show the compounds were more toxic for $HeLaP_4$ compared to MT-2 cells by MNC.

Table 3 MNC (M) AND CC_{50} (M) of the initial substances tested in MT-2 AND $HeLaP_4$

	100	101	103	105	201	205
MT-2	2.5×10^{-3}	2.5×10^{-3}	2.5×10^{-4}	2.5×10^{-4}	1.25×10^{-4}	1.25×10^{-4}
$HeLaP_4$*	6.3×10^{-13}	1.25×10^{-10}	2.5×10^{-9}	2.5×10^{-14}	1.25×10^{-7}	1.25×10^{-11}
$HeLaP_4$**	6.25×10^{-7}	6.87×10^{-6}	6.87×10^{-5}	1.25×10^{-5}	2.5×10^{-5}	2.5×10^{-8}

*MNC **CC_{50}.

Table 4 shows MNC of the derivatives of newly synthesized SQs in MT-2 cells – they are more toxic than the initial ones. It is known that free phenol group in the molecule of 8-OH quinoline and its derivatives increase their toxicity. Esterification of –OH group to 8-C of the quinoline ring declines this unfavorable effect. All initial SQs (Table 1) have acetylated hydroxyl group and are less toxic in comparison with their derivatives. The initial SQs having only one halogen substitute (201, 205) in the phenyl ring are more cytotoxic than those with additional halogen substitute (101, 105). A halogen substitute linked to 3'-C of the phenyl ring (101) confers higher cytotoxicity than that linked to 2'-C (100).

Table 4 MNC (M) of the derivatives of the initial substances in MT-2 CELLS

105H	301S	302H	303H	304B
2.5×10^{-6}	2.5×10^{-4}	2.5×10^{-6}	2.5×10^{-6}	2.5×10^{-6}

The hydrochloride derivatives are more cytotoxic than the initial substances.

Derivative 304B containing indol ring added to the phenyl ring of 105 and 205 is also more cytotoxic than the initial substances.

301S - the salt derivative of 205 does not show any changes in cytotoxicity compared to the initial substance.

Tables 5 and 6 show the results from antiviral assays for the initial substances and their derivatives. It is seen that the derivatives are more active than initial SQs (comparison of their IC_{50}). Probably, esterification of -OH group declines the chelating potency conferring the weaker inhibitory effect of initial substances.

Table 5 IC_{50} (μM) and the effect of the initial substances on HIV-1 replication in MT-2 cell, measured by cell protection (%) and inhibition of *RT* activity (%) of supernatants

	100	101	103	105	201	205	Ref. ABC
IC_{50}	*750*	*1585*	*0*	*3*	*38*	*12.5*	*5*
% cell Protection	*100**	*100***	*0*	*88.99**	*28.84***	*98***	*100*
Inhibition of *RT* in supernatants	*92.96*	*83.59*	*ND*	*81.70*	*33.91*	*27.13*	*99*

*MNC^{-3} **MNC^{-2} *MNC^{-4} ND – not done.

Table 6 Inhibition (%) of rRT by initial SQS (IN MNC)

	100	101	103	105	201	205
Inhibition (%)	89.90	*83.94*	*72.36*	*63.05*	*78.70*	*61.47*

Substances 100 and 101 show almost 100% protection of MT-2 cells infected with HIV-1 (table 5), and correlating to inhibition in RT activity in supernatants - 92.96% and 83.59%, respectively, and rRT - 89.90% and 83.94%, respectively (Tables 5 and 6). It is concluded that the mechanism of action of compounds 100 and 101 involve inhibition of HIV-1 RT.

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Substance 103 shows no inhibition of HIV-1 infectivity, correlating well to lack of inhibition of RT in supernatants. At the same time 103 directly inhibits rRT in MNC (73.36%, Table 6). The possible explanation could be that the RT in the virion is somehow 'protected' by an unknown mechanism.

Substances 105 and 205 protect cells ~ 90% (Table 5) but this effect can only partially (esp. for 205) be explained by RT and rRT inhibition (Tables 5 and 6). In 105, Cl_2 substitute is introduced in the quinoline ring (no such substitute in 205). The phenyl rings of both substances show no difference, but 205 is more cytotoxic than 105, which has a quinoline ring identical to 100 and 101 (also with low cytotoxicity).

Substance 201 protects cells ~ 30%; the RT activity in supernatants is inhibited in 33.91% while the rRT is 78.7% inhibited. This effect needs a further explanation.

Substance 105H (hydrochloride deacetylated derivative of 105) protects MT-2 cells (34.83%) but shows no RT inhibiting effect – Table 7. It is more cytotoxic than 105.

Table 7 IC_{50} (μM) and the effect of the derivatives of the initial substances (in MNC) on HIV-1 replication in MT-2 cell, measured by cell protection (%) and inhibition of *RT* activity (%) of supernatants

	105H	301S	302H	303H	304B
IC_{50}	> 0.001	*0.00225*	*0.0005*	*0.0001*	*0.0005*
% cell Protection	*34.83*	*54.77**	*78.51***	*54.75*	*69.87*
Inhibition of *RT* in supernatants (%)	*0*	*78.31*	*74.89*	*54.43*	*73.21*

*MNC x 10^{-3}. **MNC x 10^{-1}.

301S, 302H, 303H and 304B in MNC moderately protect MT-2 cells, but demonstrate inhibition of RT activity in supernatants 54–75% with higher inhibition in lower concentrations (Table 7). Except 303H, all of them show higher inhibitory effect on HIV compared to the initial substances (201 and 205). At the same time no effect on rRT was observed that clearly shows that RT is not the target of the inhibiting effect. This is not inconsistent to RT results in supernatants, because RT also measures virus replication, not only the effect on the enzyme. As far as the SQs are designed as IN inhibitors it seems possible that the derivatives inhibit the HIV-1 replication by impact on IN. Additionally, a combined effect on both RT and/or PR and IN is not excluded because of overlapping of coding regions in *pol*-gene. Further studies are planned to clarify the exact mechanism and target/s of action of the newly synthesized SQs and their derivatives. In conclusion, the derivatives of SQs described here demonstrate increased anti-HIV effect but higher cytotoxicity.

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