Journal of Labelled Compounds and Radiopharmaceuticals

Published online 14 May 2010 in Wiley Interscience (www.interscience.wiley.com) DOI: 10.1002/jlcr.1765

10th International Symposium on the Synthesis and Applications of Isotopes and Isotopically Labelled Compounds—Applications of Isotopes in Pharmaceutical Research and Development

Session 7, Tuesday, June 16, 2009

SESSION CHAIRS: CAROLEE LAVEY[a] AND DAVID HESK[b]

[a]Schering-Plough, USA
[b]Schering-Plough, USA

Abstract: The use of a Na^{125}I-Iodo-Gen system for the labelling of oxidation sensitive peptides was discussed. The question was posed as to whether the concern about reactive metabolites was warranted. In addition, the use of tritium labelling in the evaluation of a potential new nucleoside analog was discussed.

Keywords: Iodo-Gen; I-125; reactive metabolites; NVP-TAE684; nucleoside analogs; tritium labelled compounds

CONDITIONS FOR SUCCSESFUL LABELING OF OXIDATION SENSITIVE PEPTIDES BY NA[^{125}I] – IODO-GEN^{TM} SYSTEM

TOMAS ELBERT, AND IVA VESELA

Institute of Organic Chemistry and Biochemistry of Academy of Sciences of the Czech Republic, Flemingovo nam. 2, 16610 Prague 6, Czech Republic

Abstract: Very good an reproducible yields of ^{125}I-labeled peptides containing tryptophan residue were achieved when the radioiodination reaction was performed with 1 to 3 mCi of carrier free Na[^{125}I] in 1.5 mL eppendorf PP tubes precoated with 10 nmols of IODO-GEN^{TM} in phosphate buffer saline (pH=7.8). Addition of bovine serum albumin to the solutions of radiolabeled peptides greatly reduced the losses during their workup.

Keywords: IODO-GEN; Peptide; 125-I; Radiolabelling

Introduction: Labeling of peptides by carrier free ^{125}I is based on the electrophilic aromatic substitution of the tyrosyl or histidyl residue ring hydrogens by $^{125}I^{+}$. Generation of $^{125}I^{+}$ species is based on the oxidation of $^{125}I^{-}$ anion and in the case of chloro amines or chloro amides used as oxidation reagents a [^{125}I]Cl species is believed to be the active iodination reagent[1,2]. Thus the reaction milieu is oxidative and excessive oxidation reagent will damage the peptide as there are oxidation sensitive groups in peptides. The most sensitive group is the methylthio group of a methionyl residue which is oxidized to a methyl sulfoxide. Oxidation of the indole moiety in tryptophan residue leads to a rupture of the heterocyclic ring[3,4] resulting in severe peptide damage. This is usually the case of commercially available Pierce® Pre-Coated Iodination Tubes containg 50 μg (115 nmoles) of IODO-GEN^{TM} (Figure 1). The activity of carrier free Na[^{125}I] usually used in an iodination reaction is 1 mCi and it represents only 0.5 nmol. As the molecule of IODO-GEN^{TM} contains four active chlorine atoms we get a ratio of the 920 : 1 of the oxidation reagent to the $^{125}I^{-}$ equivalent. The so called Chizzonite Indirect Method for Iodination[5], described in the producer's Instructions of Use, claims to suppress the oxidation damage of proteins or peptides by generating an 'active ^{125}I species' from the Na[^{125}I] separately. The resulting solution is next added to the protein or peptide solution into a tube free of the oxidation reagent. As the excessive chloro amide reacts with water to give hypochlorous anion which is obviously present in the 'active ^{125}I species' solution, this method represents no remedy for oxidation damage of peptides. Moreover, the [^{125}I]Cl undergoes rapid hydrolysis giving iodate and iodide anions[6]. These two facts are the reason why Brown et al.[7] have found IODO-GEN^{TM} unsuitable for radioiodination of oxidation sensitive peptides. Brown et al. in the above cited paper recommended a lactoperoxidase – hydrogen peroxide system (first described in 1969 by Marchalonis[8]), as a method of choice for labeling of methionine and tryptophan containing peptides.

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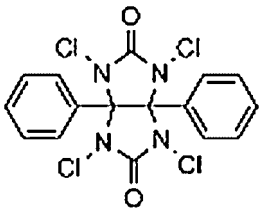

Figure 1. Structure of Iodo-Gem™.

Results and Discussion: The results obtained with lactoperoxiadase system were not very reproducible in our hands, most probably due to the instability of the enzyme. However, very good reproducibility was achieved with 1.5 mL eppendorf PP tubes precoated with 10 nmoles of IODO-GEN™ in phosphate buffer saline (pH=7.8). It is important to keep the IODO-GEN™ to peptide ratio as low as possible as demonstrated in a successful iodination of tryptofan containing peptide pentratine P-VP-141 (Figure 2).

Ac-(D)Arg-Tyr-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-NLe-Arg-Trp-Lys-Lys-$CONH_2$

penetratine P-VP-141

Figure 2. Structure of Penetratine P-VP-141.

For every new peptide, we routinely perform a 'cold' iodination experiment using an eppendorf tube containing 23 nmols (10 μg) of IODO-GEN™ and we keep NaI to peptide molar ratio 1:1. Under this condition the iodo derivatives of the corresponding peptide are formed in sufficient quantity to be visible in UV and thus we can fine tune the HPLC separation of starting peptide and its monoiodo and diiodo derivatives. When performing the 'cold' experiment with P-VP-141(see Figure 3), the 'heap' of products was not very promising and indicated extensive peptide damage. Therefore we lowered the amount of the IODO-GEN™ to 10 nmols and reversed the sequence of the reagent addition – the NaI was added prior to the peptide. Figure 4 shows the results of the ''hot'' experiment using 10 nmol IODO-GEN™ eppendorf tube, 1 mCi of Na[^{125}I] and 20 μg (8.5 nmol) of peptide. The yield of pure ^{125}I-labeled peptide was 0.45 mCi (45% based upon starting Na[^{125}I]).

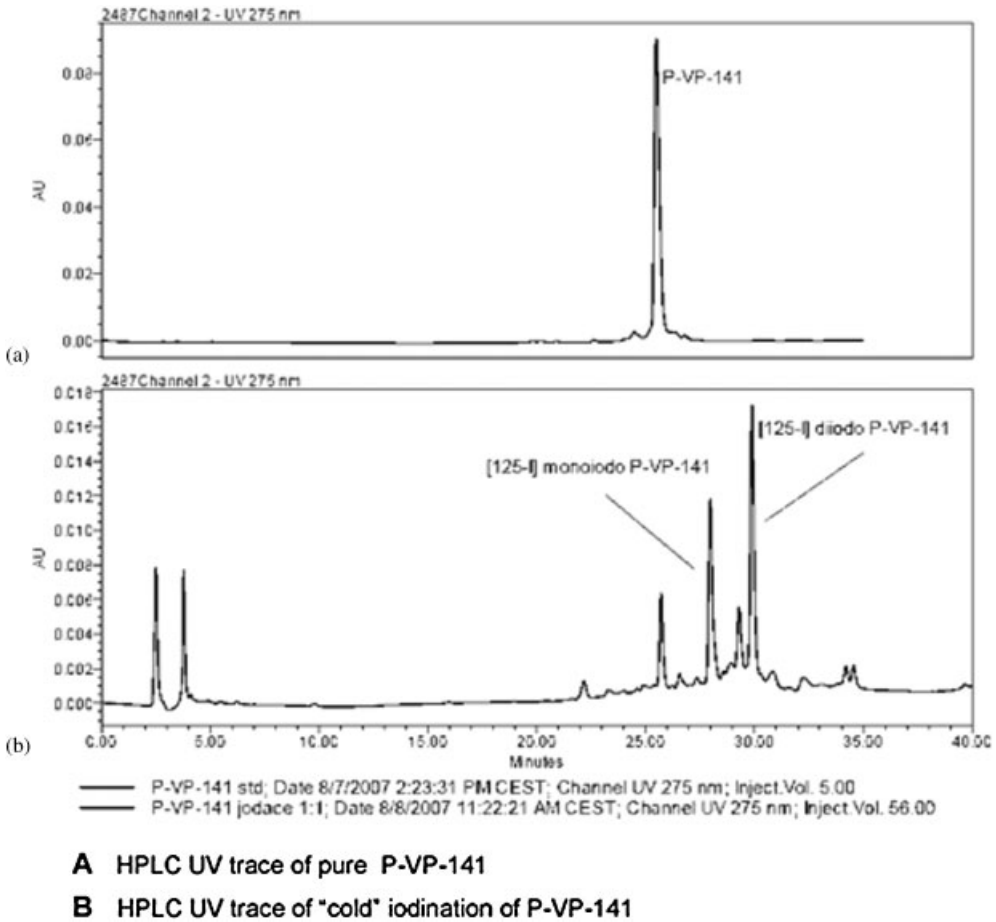

Figure 3. HPLC traces of pure P-VP-141 (a) and the ''cold'' iodination of P-VP-141 (b).

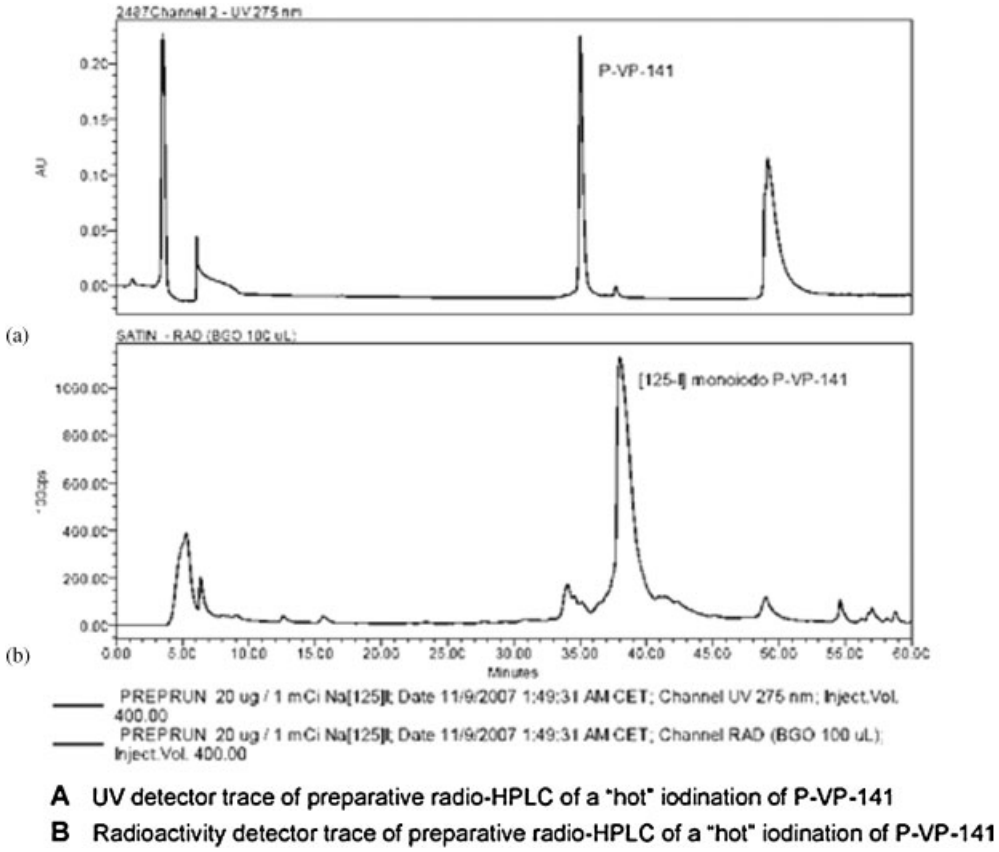

Figure 4. UV detector trace of a preparative radio-HPLC of a ''hot'' iodination of P-VP-141 (a) and a radioactivity trace of a ''hot'' iodination of P-VP-141 (b).

Oxidation sensitive peptides have another common property – a tendency to stick to the test tube wall. The problem is more severe with glass test tubes in comparison to PE and PP test tubes. Addition of bovine serum albumin (BSA) to the solutions of labeled peptides greatly reduces the problem and gives increased yields. Thus HEPES-BSA buffer was always added to the radioiodination reaction mixture prior to its injection onto the HPLC column. Whenever possible the HEPES-BSA buffer was also added to fraction collector tubes prior to preparative HPLC run[9].

For low concentrations of some peptides even this measure fails. We observed such discrepancies while routinely assaying the activity of a ^{125}I-labeled peptide with this amino acid sequence:

Tyr-Ser-Ala-Leu-Leu-Arg-Ser-Ile-Pro-Ala-Pro-Ala-Gly-Ala Ser-Arg-Leu-Leu-Leu-Thr-Gly-Glu-Ile-Asp-Leu-Pro.

As usual we diluted our peptide-HEPES-BSA stock solution with some HEPES-BSA and assayed an aliquot a 0.87 μCi/mL. After 30 minutes we repeated the measurement with another aliquot of the same solution and read 0.67 μCi/mL. 20 minutes later, the activity concentration dropped to 0.59 μCi/mL. However, when we diluted the peptide stock solution with a mixture of methanol – water – trifluoracetic acid 60:40:1 (v:v:v) the activity concentration reading was 1.4 μCi/mL, i.e. 160% of the reading in HEPES-BSA. Therefore dilution in this methanol – water – trifluoracetic acid mixture is now used in our laboratory as a standard protocol for activity assays of ^{125}I-labeled peptides.

Although the oxidation of the methylthio group of methionyl residues to the corresponding methylsulfoxide group could not be suppressed completely under these conditions, it was comparable to the extent of methylthio group oxidation by the lactoperoxidase - hydrogen peroxide system. In the case of CART(61–102) peptide (Figure 5) it turned out that the oxidation of the methionyl residue to sulfoxide did not influence its receptor binding constant.

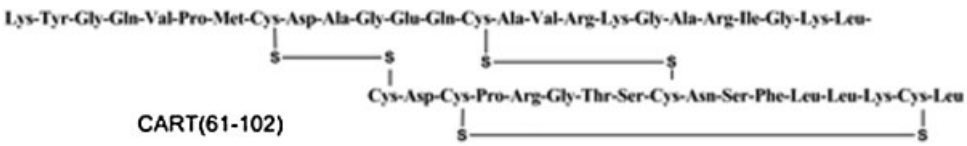

Figure 5. Structure of CART (61-102) peptide.

Conclusions: To obtain the best results in the labeling of oxidation sensitive peptides by radionuclide ^{125}I, the following guidelines should be observed:

- Peptide : IODO-GEN^{TM} molar ratio around 1:1.
- Peptide : Na[^{125}I] molar ratio around 20:1 (to suppress the formation of diiodo derivative).

- Na[^{125}I] must be added to the iodination eppendorf tube prior to the peptide addition.
- Before the transfer of the reaction mixture to an HPLC column, the same volume of the HEPES-BSA buffer must be added.
- Whenever possible the HEPES-BSA buffer must be added to fraction collector tubes (a volume equal to the volume of a fraction).

Experimental: The HPLC analysis was performed on a system consisting of a Waters Delta 600 Pump and Controller, Waters 2487 UV detector and a Ramona radio chromatographic detector from Raytest with interchangeable fluid cells. For preparative runs the BGO cell was used, for analytical runs the column effluent was mixed with Zinsser Quickszint Flow 302 cocktail in a 1:4 ratio. Data was collected and processed using Empower 2.0 software. A Synergi 4 μ Fusion-RP 80, 250 × 3 mm HPLC column equipped with SecurityGuard^{TM} (both from Phenomenex) was used.
HPLC eluents:

A=water with 0.1% of trifluoroacetic acid
B=acetonitrile (gradient grade from Merck) with 0.1% of trifluoroacetic acid

Activities were measured on a Wallac 1470 Gamma Counter (Perkin-Elmer). Evaporations were done using a CentriVap Concentrator from Labconco. The following buffers were used: PBS=100 mM phosphate buffer, pH=7.8, containig 150 mM NaCl HEPES-BSA=20 mM HEPES buffer, pH=7.4, containing 1 mg /mL of bovine serum albumin.

Carrier free Na[^{125}I] was purchased from MP Biomedicals, Inc.

Coating of Eppendorf Tubes with IODO-GEN^{TM}

A solution of IODO-GEN^{TM} in dry dichloromethane (0.1 mg/mL) was prepared. Corresponding aliquots of this solution were pipetted into 1.5 mL conical eppendorf tubes, the solvent was evaporated (40°C, 15 min.) and the tubes were stored over silica gel at −20°C. No loss of activity of the coated tubes was observed even after three years of storage.

"Cold" iodination of penetratine P-VP-141

An eppendorf tube containing 23 nmols of IODO-GEN^{TM} was first washed with 100 μL of PBS. Then 200 μL of PBS was added followed by 20 μg (8.5 nmol) of penetratine P-VP-141 (20 μL). The solution of 8.5 nmol of NaI in 8.5 μL of water was added and the reaction mixture was vigorously shaken for 15 minutes. An aliquot (100 μL) of the reaction mixture was analyzed by HPLC.

Iodination of penetratine P-VP-141by Na[^{125}I]

An eppendorf tube containing 10 nmols of IODO-GEN^{TM} was first washed with 100 μL of PBS. Then 200 μL of PBS was added followed by 1 mCi of carrier free Na[^{125}I]. Penetratine P-VP-141 (20 μg, 8.5 nmol, in 20 μL of water) was added and the reaction mixture was vigorously shaken for 15 minutes. The HEPES-BSA buffer (200 μL) was added and the whole reaction mixture was applied to an HPLC column. Gradient elution was performed from 20% of B to 40% of B in 30 minutes at 0.5 mL/min. HEPES-BSA buffer was added to collector tubes prior to preparative run start.The pooled fractions eluted between 37–40 minutes to give 0.45 mCi (45%) of [^{125}I]monoiodo P-VP-141. Radiochemical purity was analyzed using the same column-solvent system and was 99% . Aliquots of [^{125}I]monoiodo P-VP-141 were distributed to eppendorf tubes, evaporated to dryness (CentriVap heating OFF, overnight) and stored at −20°C.

Acknowledgement: This work was performed as part of a research project of the Institute of Organic Chemistry and Biochemistry No. Z40550506.

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WHEN SHOULD WE WORRY ABOUT COMPOUNDS WITH POTENTIAL REACTIVE METABOLITES?

FREDERICK R. KINDER, JR.

Novartis Institutes for Biomedical Research, 250 Massachusetts Ave., Cambridge, MA 02139, USA

Abstract: Drug-induced hepatic injury is the major reason for drug withdrawal and late stage failure of drug candidates. The majority of compounds with clinically relevant signs of hepatotoxicity form reactive intermediates. Reactive intermediates, usually

electrophiles or free radicals, may form during metabolic clearance of compounds. These unstable metabolites may then react spontaneously with endogenous nucleophiles like glutathione, DNA, or proteins. While toxicity in man involving covalent adduct formation is not always predicted from preclinical studies, we are challenged to eliminate this risk without loss of development time or needless compound termination.

A number of early profiling activities to assess the potential of covalent adduct formation are available to drug discovery scientists. These include *in silico* tools, preliminary non-radioactive *in vitro* methods, more thorough *in vitro* methods using radiolabelled compound, and animal studies. Results from these profiling activities may be used to alter the course of medicinal chemistry lead optimization efforts or support early "No Go" decisions for compounds that form reactive metabolites. Several examples will also be included in the discussion.

Keywords: reactive metabolites; NVP-TAE684; ALK

Introduction: Termination of drugs in late clinical development can be disastrous. Attrition during early drug development or even during compound lead optimization is more desirable than later during IND-enabling studies or clinical trials. Of course, every compound needs to be evaluated as a special case so that every early attrition risk uncovered doesn't result in premature termination. Contributors to attrition identifiable during lead optimization or early preclinical development include: poor potency and/or selectivity, narrow therapeutic index in animal models, undesirable physicochemical properties, technical issues (e.g. synthetic complexity), poor pharmacokinetic properties, and formation of reactive metabolites. This last attrition risk is of particular concern since the vast majority of compounds with clinically relevant signs of hepatotoxicity form reactive intermediates. Furthermore, hepatic injury is the major reason for drug withdrawal and late stage failure of drug candidates.[1]

Results and Discussion: Reactive intermediates are formed during metabolic clearance of drugs. Occasionally these metabolic reactions produce unstable/reactive intermediates which may react spontaneously with endogenous nucleophiles including small molecules (e.g. glutathione) or macromolecules (e.g. protein, peptides or DNA). Examples of reactive metabolites include epoxides, iminium ions, nitrenium ions, michael acceptors, aldehydes, S-oxides, carbenes, and acylglucuronides (see Figure 1).

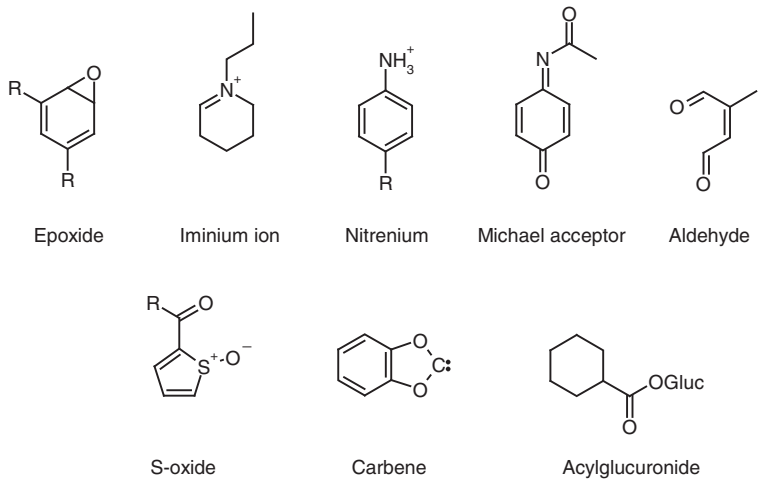

Figure 1. Examples of reactive metabolites.

The consequences of forming reactive intermediates are fourfold: (1) potential for genotoxicity as a result of forming covalent DNA adducts, (2) potential for immune-mediated adverse drug reactions as well as general off-target toxicity as a result of covalent binding to cellular proteins, (3) potential for oxidative stress as a consequence of forming reactive oxygen species, and (4) general glutathione (GSH) depletion.

Consider the case of NVP-TAE684, an anaplastic lymphoma kinase (ALK) inhibitor developed for the treatment of patients with NMP-ALK expressing tumors.[2] NVP-TAE684 had an excellent preclinical anti-tumor profile including impressive anti-lymphoma activity in several mouse efficacy models. The molecule had an acceptable physicochemical and early PK profile and was considered a good candidate for further preclinical development including GLP toxicology studies and a rat ADME study. However, NVP-TAE684 was terminated prior to the start of Phase I for the following reasons: (1) certain non-monitorable and irreversible toxicological findings in the liver, pancreas, heart, lung, bone marrow, thymus, and hematological findings in rats and dogs, (2) signals for geno- and cardiotoxicity *in vitro* (polyploidy and hERG inhibition), (3) signal for glucose intolerance, and (4) significant formation of reactive metabolites observed in feces (15% of dose) and 11–13% of the dose remained in the carcass 168 h after a single iv or oral dose, which may be covalently bound reactive metabolites.

The formation of reactive metabolites from NVP-TAE684 undoubtedly contributed to the numerous tox. findings observed in rats and dogs. The chemical structure of tritiated NVP-TAE684 and one potential pathway to a covalent adduct can be found in Scheme 1. Oxidation of the 1,4-diaminobenzene ring could provide a quinone diiminium intermediate which could undergo nucleophilic attack by GSH or cysteine. In addition, the rat ADME study with [^{3}H]NVP-TAE684 revealed extensive metabolism including oxidations, N-dealkylkations, N-methylations, N-acetylations, and numerous glutathione and cysteine adducts. More than 50 metabolites were observed in the feces.

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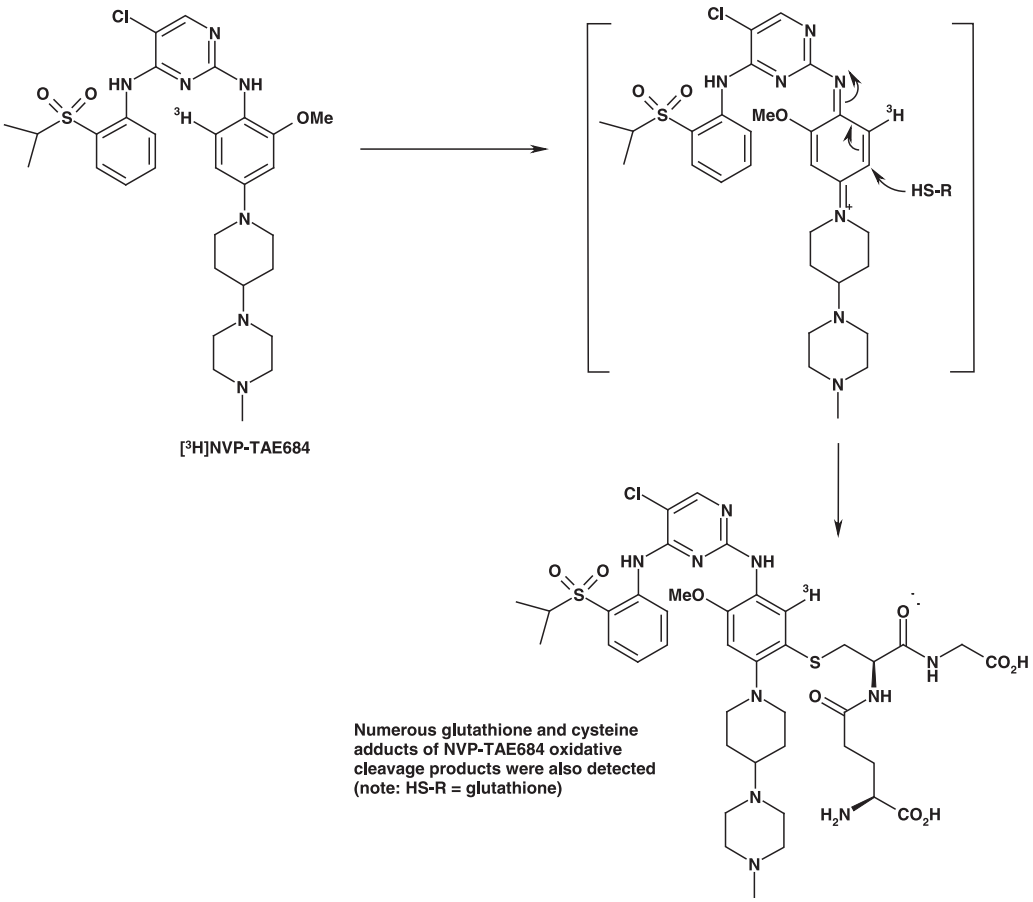

Scheme 1. Structure of [^{3}H]NVP-TAE684 and potential pathway to formation of glutathione adduct.

Given the case with NVP-TAE684 and other projects with similar fates, Novartis Institutes of Biomedical Research (NIBR) have implemented several early practices to avoid the risk of reactive metabolites during lead optimization and/or as early as possible during preclinical development.

Several 'truth-telling' activities can influence medicinal chemistry efforts during lead optimization. These are: (1) In Silico Tox Check,[3] (2) in vitro metabolite identification across species using non-radiolabelled compounds, and (3) screening for reactive intermediates and/or metabolites using GSH trapping experiments. Generally, compound series with relatively high amounts of GSH adducts should be avoided. However, it should be noted that GSH adduct levels cannot be readily quantified, not all reactive compounds react with GSH, and the true relationship with GSH adducts and liver (and other organ) toxicity has not been unequivocally established.

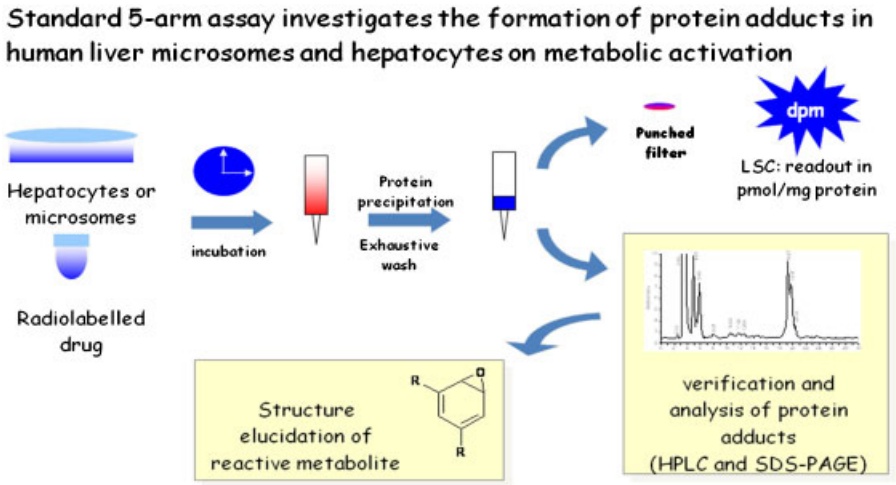

Figure 2. 5-arm assay principles.

Once a compound has been selected for early development, the risk for liver toxicity as a consequence of formation of reactive metabolites can be assessed using radiolabelled compounds in the '5-arm' assay. The 5-arm assay investigates the formation of protein

adducts in human liver microsomes (HLM) and hepatocytes following metabolic activation (see Figure 2). Briefly, the radiolabelled drug is incubated for 1–3 hours at 37°C with human liver microsomes or human hepatocytes, protein is precipitated and exhaustively washed, punched filters are prepared and analyzed for radioactivity indicating covalent binding of the radiolabelled drug to proteins. If necessary, the precipitate is analyzed using HPLC and SDS-PAGE to identify the structure of the reactive metabolite.

The individual arms are as follows: (1) HLM + GSH as the negative control, (2) HLM + NADPH to investigate metabolic activation, (3) second arm + UDPGA to investigate the influence of glucuronidation, (4) third arm + GSH in order to understand the influence of GSH trapping, and (5) hepatocytes (0.5×10^6 cells/mL). Values of >100 pmol/mg in the second and/or third arm or > 50 pmol/10^6 cells in the fifth arm trigger further mechanistic studies to determine which proteins are covalently modified by the drug candidate. Decisions to continue development on compounds that score positively in the 5-arm assay are made based on potential target patient phenotype/risk factors, projected effective dose in man, predicted PK in man, and results from early toxicology studies.

Summary: Detection of protein adducts after metabolic activation of a drug indicates a structural liability that can lead to adverse drug reactions. Protein adducts can mediate toxicity, but may not always lead to toxicity in the clinic. Given the high uncertainty of the clinical relevance at such an early stage in preclinical development, structural elements of drug candidates that contribute to positive 5-arm assay scores should be avoided in future lead optimization efforts. Finally, the clinical relevance of adduct formation depends largely upon: (1) the extent of covalent adduct formation, (2) the dose and exposure predicted in patients, (3) the relevance of the actual proteins being covalently modified, and (4) the predicted susceptibility within the intended patient population.

Acknowledgements: Special thanks go to Peter End, Michael Kiffe, Jeffrey Cramer, and Alan Collis for their help in the preparation of this presentation.

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IN VITRO AND *IN VIVO* RADIOMETRICAL STUDIES FOR EVALUATION OF NEW NUCLEOSIDE ANALOGUE BEHAVIOR

LIDIA MATEI,[a] IONELA NEAGOE,[b] C. POSTOLACHE,[a] V TANASE,[c] G. BUBUEANU,[a] AND GINA MANDA[b]

[a]National Institute of Research and Development for Physics and Nuclear Engineering "Horia Hulubei", 407 Atomistilor street, Magurele, Romania.
[b]"Victor Babes" National Institute of Pathology, Bucharest, Romania.
[c]National Institute of Chemical and Pharmaceutical Researches Institute, Bucharest, Romania

Abstract: We investigated the anti-neoplastic activity of a novel pyrimidine nucleoside analog, U-34, in which the sugar moiety was modified by replacing the furane ring with a functionalized oxabicyclo [3.3.0]octane fragment and the naturally occurring pyrimidine uracil was used as the base. Our experimental data showed that U-34, at high concentrations, was cytotoxic for Jurkat lymphoblasts and significantly inhibited tritium-labeled uridine and thymidine uptake. Although lower doses of U-34 were not cytotoxic or cytostatic for Jurkat cells, they limited tritium-labeled uridine and thymidine incorporation, without altering cell viability and proliferation capacity. U-34 exerted these effects by entering tumor cells, but we may not rule out that interaction with nucleoside receptors may be partly responsible for the observed action of U-34 on Jurkat cells. Using Wistar rats and a tritium-labeled U-34, we showed that the nucleoside analog accumulated rapidly and persistently in various organs and blood. The methodological approach, combining conventional cellular biology test and radiometrical methods, proved to be valuable for characterizing the in vitro mechanism of action of U-34 on cancer cells and for assessing its biodistribution in animals.

Keywords: nucleoside analogs; anti tumour activity; tritium; radiometrical studies

Introduction: Cancer is one of the leading causes of death and represents a tremendous burden on patients, families and societies. Nucleoside analogues are an effective class of chemotherapeutic drugs in cancer, mainly addressing leukaemia, but their clinical use is unfortunately limited by severe side-effects and acquired drug resistance.[1] Accordingly, a huge research effort is currently focused on the development of new, more efficient and more selective antitumor nucleoside analogues, with limited side-effects. In this paper we describe several experimental findings regarding the biologic action of the novel nucleoside analog U-34, in which the sugar moiety was modified by replacing the furane ring with a functionalized oxabicyclo [3.3.0]octane fragment and using as base the naturally occurring pyrimidine, uracil. In our experimental in vitro and in vivo models we used both conventional cellular biology tests and radiometrical methods for characterizing the in vitro mechanism of action of U-34 on cancer cells and for assessing the biodistribution of the nucleoside analog in animals. The biodistribution of U-34 was evaluated using the tritium labelled[2] [5-T]-U-34 as a tracer.

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Experimental:

Reagents: RPMI 1640 medium, fetal bovine serum and antibiotic-antimycotic solution were purchased from Sigma-Aldrich. CellTiter 96 AQueous One Solution Cell Proliferation Assay and CytoTox 96 Non-Radioactive Cytotoxicity Assay were from Promega Corporation. Soulene 350 and Hionic Fluor liquid scintillation cocktail were provided from Perkin Elmer.

Nucleoside analogue: We investigated the novel oxabicyclo[3.3.0]octane nucleoside analogue U-34, in which the sugar moiety was modified by replacing the furane ring with a functionalized oxabicyclo [3.3.0]octane fragment and the naturally occurring pyrimidine uracil represents the base (Figure 1).

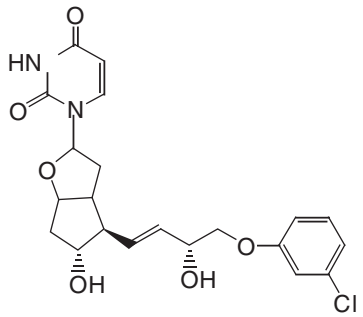

Figure 1. The structure of the novel oxabicyclo[3.3.0]octane nucleoside analogue U-34.

The synthesis, the physical and chemical properties of U-34 were previously described by us.[3] For cellular in vitro studies, U-34 was initially dissolved in dimethyl sulfoxide (DMSO) and was further diluted in cell culture medium. The final concentration of DMSO in experimental systems was less than 0.1% and did not significantly alter cellular metabolism and functionality.

Tritiated compounds: Tritium-labeled U-34 (U-34-T) in ethanoic solution, with radioactive concentration of 16 MBq/ml and 984 GBq/mmol specific activity.

Tritium labeled Uridine-5-T in aqueous solution, with radioactive concentration 25,9 MBq/ml and 851 GBq/mmol specific activity.

Tritium-labeled Thymidine-Me-T in aqueous solution, with radioactive concentration of 22.9 MBq/ml and 905 GBq/mmol specific activity.

The tritium-labeled compounds were synthesized at the Tritium Laboratory of the National Institute for Physics and Nuclear Engineering.

Cells: We investigated standardized Jurkat cells which are human leukemia T lymphoblasts. The cell line was purchased from the European Collection of Cell Cultures (ECACC, UK) and was maintained in culture according to the procedure provided by the depositor.

Cell counts and viability: Cells were counted in a Burker-Turk hemocytometer. Cellular viability, scored by trypan blue exclusion, was higher than 98% before applying cell treatment with nucleoside analogues.

Cellular parameters: *Cell viability/multiplication* was measured by the tetrazolium salt (MTS) reduction test, using CellTiter 96^{R}, AQueous One Solution Cell Proliferation Assay (CellTiter 96R AQueous One Solution Cell Proliferation Assay Technical Bulletin ♯TB112, Promega Corporation). This test gives information about the amount of metabolically active cells in culture.

Cell death by necrosis was evaluated as disruption of plasma membrane integrity and measured by the LDH release test, using Cytotox96 Non-Radioactive Cytotoxicity Assay (Technical Bulletin ♯TB163, Promega Corporation).

Nucleoside uptake by cells was evaluated using the tritium-labeled uridine or thymidine incorporation method.[4,5] Cancer cells were incubated with tritium-labeled uridine or thymidine in the last 5 h of cultivation. This method is mainly a measure of RNA and DNA synthesis, respectively. If cells are treated with uracil-based nucleoside analogs, like U-34, the intensity of uridine uptake might also reflect disturbances induced by the drug at the level of cellular nucleoside receptors or metabolism.

U-34 uptake by Jurkat cells was measured using the above described tritium-labeled variant, U-34-T. Cells were treated for 24 h with U-34-T, in absence and presence of the non-labeled compound, and were further processed for radioactivity measurement as described by Manda et al. [5]

Biodistribution of U-34-T. For in vivo studies, male, young adult Wistar rats were used. Animals were provided by the Animal Care Unit of Victor Babes Institute of Pathology. Six Wistar rats were inoculated intraperitoneally U-34-T (7.4MBq /rat). Animals were kept in standardized conditions of food and medium in the Animal Care Unit from Life and Environmental Physics at the National Institute for Physics and Nuclear Engineering. At 6 and 21 hours after inoculation, series of 3 rats were dissected and various biologic samples were harvested and weighed: submandibular lymph nodes (SG), inguinal lymph nodes (IG), brain, kidney, spleen, liver, blood, heart, adrenal gland. Biologic samples were digested with Soulene 350 and gently heated for 3 h at 55°C. After digestion, 10 ml of Liquid Scintillation Cocktail Hionic Fluor was added to each sample. Sample radioactivity was measured in a TRICARB 2800 TR Liquid Scintillation Counter. Luminescence and quenching corrections were applied. The radioactivity was expressed as Bq/g of tissue.

Statistics. Results were expressed as mean $\pm$ standard error of the mean (SEM). All statistical calculations were performed with Analyse IT software. The effect of U-34 on cellular parameters was calculated as a ratio between (parameter value in presence of U-34) and (parameter value in presence of DMSO and in absence of U-34).

Results and discussion: The effects exerted in vitro by U-34 on Jurkat lymphoblasts

We investigated the effects exerted in vitro by the novel nucleoside analog U-34 on the capacity of Jurkat lymphoblasts to incorporate exogenous nucleosides (tritium-labeled uridine or thymidine), in relation to the effect of U-34 on cell viability/multiplication.

Cells were cultivated for 24 h and 48 h in the presence of (1–100)μM U-34, starting from an initial cellular density of 10^5 cell/mL (Figures 2a and b).

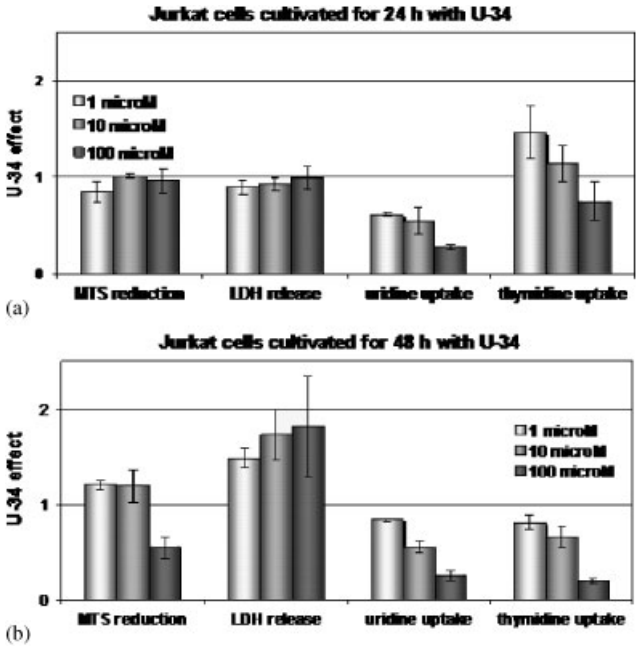

Figure 2. The effects exerted in vitro by U-34 on Jurkat lymphoblasts. Cell viability/multiplication was measured by the MTS reduction test and membrane integrity by the LDH release uptake. Uridine and thymindine uptake was measured using tritium-labeled variants. Results are presented as mean ± SEM for 3 experiments: (a) cellular parameters after 24 h cultivation with U-34 and (b) Cellular parameters after 48 h cultivation with U-34.

The effect of U-34 on cell viability/multiplication was evaluated by correlating the amount of viable cell in culture (the MTS reduction test) with their membrane integrity (the LDH release test). This approach allows highlighting distinctively cytotoxic versus cytostatic effects of drugs. Thus, reduction of the number of metabolically active cells, accompanied by breakdown of membrane integrity is an indicative of a cytotoxic effect. Meanwhile, the reduction of the number of metabolically active cells, without major alteration of membrane integrity, points out a cytostatic action of the drug.

Preliminary results published by us[3] indicated that 30 μM U-34 exerted a cytotoxic effect on human Jurkat lymphoblasts in 48 h-cultures, whilst not significantly affecting the viability of human U937 cells presenting characteristics of neoplasic proliferating monocytes. Therefore, in this study we extended the concentration domain towards higher doses (100 μM) that may also impact on other cancer cells, besides Jurkat lymphoblasts.

Our experimental data showed (Figure 2) that U-34 exerted cytotoxic effects on Jurkat cells at 100 μM, but not at lower concentrations (1 and 10 μM). This is a late effect, noticed in 48 h-cultures, but not in 24 h-cultures. Interestingly, 1 μM and 10 μM U-34 disrupted membrane integrity at 48 h, as shown by the enhanced LDH release, but did not reduce the number of metabolically active cells.

Albeit not affecting cellular viability in 24 h- and 48 h-cell cultures, 1 and 10 μM U-34 inhibited uridine uptake by Jurkat cells even early, at 24 h. This effect was observed also at 48 h of cultivation, thus proving to be a persistent one. Higher concentrations of U-34 (100 μM) induced a severe inhibition of uridine uptake, both in 24 h- and in 48 h-cultures. We also showed that inhibition of uridine uptake by Jurkat cells is an early effect of U-34, exerted even in short-term exposure of cells (6 h) to the nucleoside analog (Figure 3). Moreover, we highlighted that U-34 inhibited uridine uptake even when cytotoxic effects were not recorded (Figure 2). As such, our results indicated that inhibition of uridine uptake did not always trigger cell death or cell growth arrest.

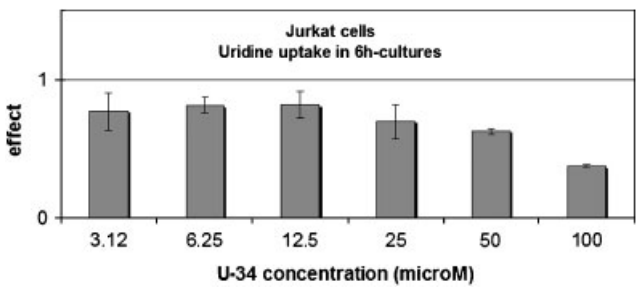

Figure 3. The effects exerted in vitro by U-34 on tritium-labled uridine uptake by Jurkat lymphoblasts in 6 h-cultures. Results are presented as mean ± SEM for 3 experiments.

At high concentration (100 μM), U-34 exerted an early moderate inhibitory effect on thymidine uptake by Jurkat cells (at 24 h), which was significantly reinforced at 48 h. Surprisingly, low concentrations of U-34 (1 μM) tended to increase thymidine uptake by Jurkat lymphoblast at 24 h, which was not accompanied an increase of the number of metabolically active cells. This effect did not reflect cycle arrest in the S-phase (data not shown). We cannot rule out depletion of intracellular thymidine stores, which could trigger the enhanced thymidine uptake observed in 24 h-cultures, followed by a decrease in 48 h-cultures.

According to our results, high concentrations of U-34 (100 μM) may dramatically alter both thymidine and uridine uptake by Jurkat lymphoblasts, this inhibitory action being more profound than the concomitant decrease of the viable cells number. A similar action pattern was recorded when treating U937 malignant cells with 100 μM U-34.

Incorporation of U-34 into Jurkat lymphoblasts. The results presented above showed that the effects of U-34 on uridine and thymidine uptake by Jurkat cells did not correlate with the effects on cell viability/multiplication. These findings raised the question whether U-34 entered into cells or it only interacted with surface nucleoside receptors, thus hindering uridine uptake.

Therefore, we investigated the uptake of small amounts of U-34 by Jurkat lymphoblasts, using the tritium-labeled form of the nucleoside analog. As shown in Figure 4a, U-34-T is incorporated into Jurkat cells following a dose-response curve. U-34-T is progressively incorporated into Jurkat lymphoblasts in the first 3–4 hrs of exposure and thereafter a steady equilibrium is apparently achieved (Figure 4b)

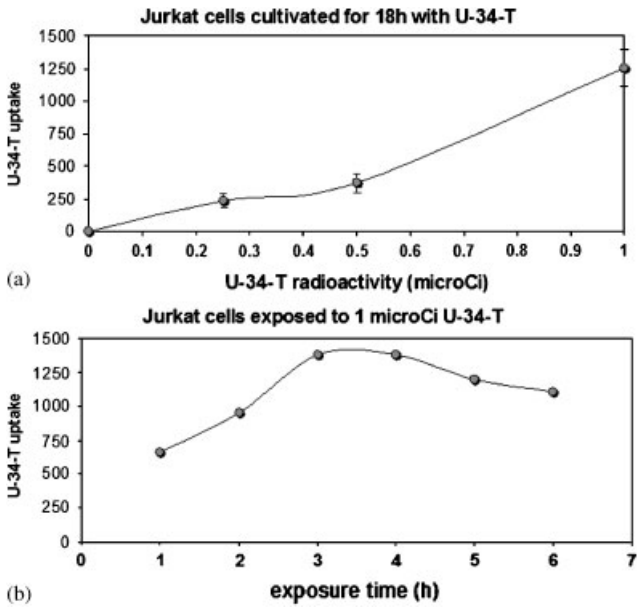

Figure 4. Uptake of tritium-labled (U-34-T) by Jurkat lymphoblasts (10000 cells). Results are presented as mean ±SEM for triplicate sample: (a) dose-response profile and (b) time-response profile.

Another aspect regarding the uptake of U-34 by Jurkat cells is worth mentioning. As compared to uridine, the radioactivity associated with U-34-T, incorporation into cells, is 10–20 fold lower (data not shown). Accordingly, we may presume that Jurkat cells are endowed with a particular ability to incorporate uridine for constitutive proliferation, but this mechanism cannot fully sustain the uptake of the nucleoside analog.

Preparing in vivo testing of U-34 in animal models: After investigating in vitro the anti-neoplastic action of U-34 on various leukemia cell lines (the action on Jurkat lymphoblasts is described in this paper), several experiments were performed for preparing in vivo protocols in animal models (Wistar rats bearing peritoneal epithelial Walker carcinoma cells in ascitic form).

We point out herein the studies regarding the biodistribution of U-34-T, 6 h and 21 h after intraperitoneal inoculation in normal Wistar rats. We have chosen this route of administration for U-34 as we will further perform experiments in animals bearing Walker cancer cells in the peritoneum.

Various biological samples were harvested and processed for assessing radioactivity: bowel, liver, spleen, kidney, heart, brain, submanidbular (SG) and inguinal lymph nodes (IG), adrenal gland and blood.

Due to the intraperitoneal route of administration, U-34-T is highly concentrated at 6 h in the bowel, but radioactivity decreases after 21 h (Figure 5).

U-34-T also accumulated rapidly in liver at 6 h and a marked decrease of radioactivity was observed at 21 h. Lower, but significant radioactivity was detected at 6 h in all the other investigated organs and in blood. In this cases, radioactivity was detected almost unchanged 21 h after U-34-T inoculation (Figure 6).

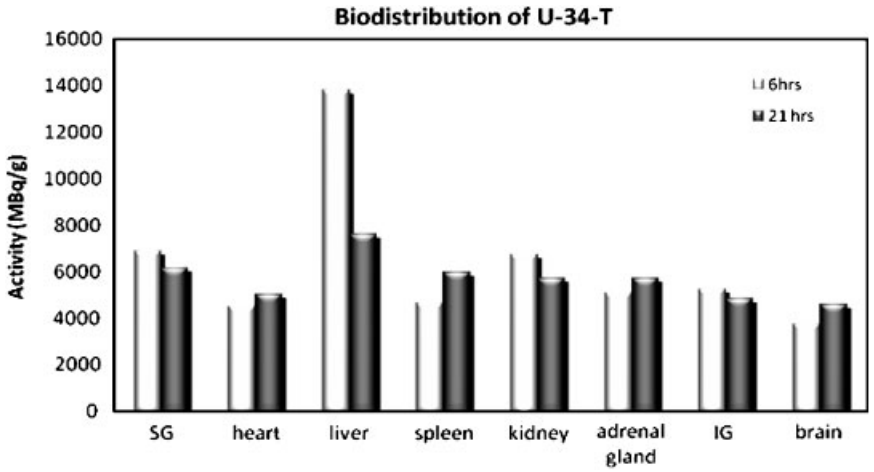

Figure 5. Biodistribution of U-34-T in organs and blood of Wistar rats at 6 and 21 h after intraperitoneal injection.

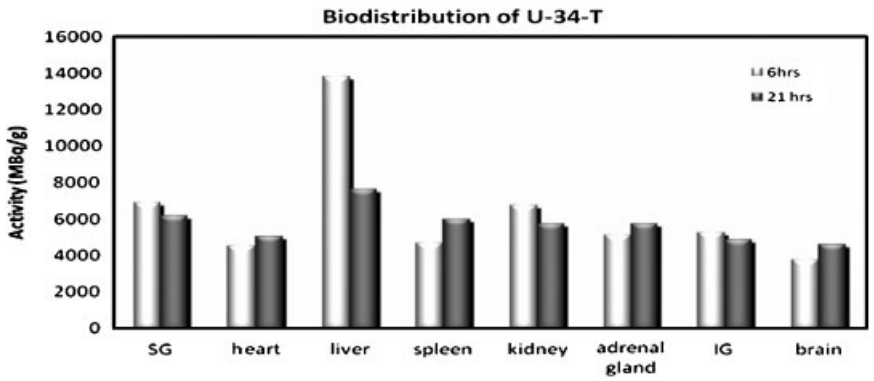

Figure 6. Biodistribution of U-34-Tin organs and blood of Wistar rats at 6 and 21 h after intraperitoneal injection.

As such, the decrease of radioactivity detected in bowel and liver at 21 h when compared to 6 h, is apparently not related to difusion of U-34-T towards other organs (at least not to the tested ones). As urine was not collected in this preliminary experiment, this study cannot provide information about the excretion rate of U-34.

Moreover, considering that only small amounts of U-34 were detected in secondary lymphoid tissues and in blood, U-34 may have in vivo, at least theoretically, only minimal effects on the immune response.

It is worth mentioning that U-34 accumulated in the brain were it is retained for at least 21 h. These data sustain our observation that Wistar rats treated with U-34 in the concentration range (10–40 mg) per kg body mass showed agressiveness for at least 28 days. Albeit being nervous, the animals treated with U-34 presented a normal health status (data not shown).

Conclusions: Our experimental data showed that the novel nucleoside analog U-34, at high concentrations, was cytotoxic for Jurkat and U937 lymphoblasts and significantly inhibited uridine and thymidine uptake. Although lower doses of U-34 were not cytotoxic for Jurkat cells, they limited uridine and thymidine incorporation. Probably, the degree of inhibition was not sufficient for altering cell viability and proliferation capacity. U-34 exerted these effects by entering cancer cells, but we may not rule out that interaction with nucleoside receptors[6] may be partly responsible of the observed action of U-34 on Jurkat lymphoblasts. Jurkat cells are able to incorporate uridine for constitutive proliferation, but this mechanism cannot fully sustain the uptake of the nucleoside analog. Further investigation is required for establishing the role of nucleoside receptors in mediating the effects of U-34 in Jurkat and other cancer cells. As shown using Wistar rats and tritiated U-34, U-34 accumulated rapidly and persistently in various organs and blood, when intraperitoneally delivered. It is noteworthy that protection mechanisms were seemingly acting in liver, at least by limiting drug concentration after an initial phase of accumulation. The presence of U-34 in all the investigated organs leads towards further systematic studies regarding the organ-specific effects of U-34. Such an approach aims to establish the toxicologic profile of U-34, and also to identify the most probable organs/tissues U-34 is addressing and where the nucleoside analog may exert efficient anti-neoplastic effects. The methodological approach, combining conventional cellular biology test and radiometrical methods, proved to be valuable for characterizing the in vitro mechanism of action of U-34 on cancer cells and for assessing its biodistribution in animals.

Acknowledgments: Research was financially supported by the Excellence Research Program, Grant 36/2005. We thank Mr. Gheorghe Savi from Victor Babes National Institute of Pathology for providing animals for in vivo studies.

The authors express their gratitude to the International Atomic Energy Agency (IAEA) for official sponsorship of present the study at The 10th International Symposium of the International Isotope Society held on June 14–18, 2009 in Chicago, IL, USA.

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