Comparison of Bioactivities of 5-Fluoro, 5-Iodo, 5-Iodovinyl, and 5-Fluorovinyl Arabinosyl Uridines against SR-39 TK-Transfected Murine Prostate Cancer Cells

Chi-Shiun Chiang, Ching-Fang Yu, Li-Wu Chiang, Shao-Wei Chen, Jem-Mau Lo, and Chung-Shan Yu*

Department of Biomedical Engineering and Environmental Sciences, National Tsing-Hua University; No. 101 Sec. 2, Guang-Fu Rd., Hsinchu 30013, Taiwan. Received September 10, 2007; accepted October 24, 2007

A cell survival assay of the four arabinosyl uridine analogs with functionalities of 5-fluoro, 5-fluorovinyl, 5-iodo, and 5-iodovinyl as potential positron-emitter tagged probe for monitoring cancer gene therapy were performed. Cytotoxicities of 5-fluoro-, 5-iodo-, 5-fluorovinyl, and 5-iodovinyl arabinosyl uridines against SR-39 thymidine kinase transfected murine prostate cancer cells have been evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. None of them showed significant bioactivity. A *syn* conformation derived from intra-hydrogen bonding was suggested for the unfavorable interaction and diminished bioactivity.

Key words arabinosyl uridine; SR-39 TK; fluoro; iodo

Due to the potency of gene-engineered techniques in drug development, various mutants of herpes simplex virus thymidine kinase (HSV-1 TK) have been generated. Among them, SR-39,[1] SR-26,[1] A-167Y,[2] A-167F[3] and A168H[3] have found to possess the highest affinity for acylic purine nucleosides *e.g.* Gancicrovir (GCV) or acyclovir (ACV). These bioactive mutants were screened from a pool of enzymes created by systematic swapping of the amino acid residues between 159 to 184 using wild-type HSV TK as a template. Of all the mutants being tested, residues 167 and 168 located in the active site have shown to play a critical role in tuning the enzyme catalytic activity. For instance, the affinity for purine- but not pyrimidine-containing nucleosides can be improved by replacing the natural alanine-167 or alanine-168 with aromatic-containing amino acids. Therefore, thymidine served as a natural substrate for human TK but exerted poor affinity for the mutant TKs, a character that could prevent from the undesired uptake during gene therapy. In spite of its improved substrate selectivity, the catalytic efficiency of mutant forms showed an overall reduction of 20—60-fold compared to the wild type.

Although acyclic purine nucleosides rather than pyrimidine nucleosides generally show better substrate-specificity toward the mutant enzyme SR-39 TK, a 5-substituted pyrimidine nucleoside, 2′-fluoro-5-ethyl-arabinosyl uridine (FEAU), does not apply in this category.[4] Notably, not only SR-39 TK but also wild type HSV TK could efficiently phosphorylate FEAU. This unexpected activity revolutionized the current understandings that the unfavorable steric hindrance between the side chain at the 5-position of FEAU and the bulky aromatic ring of the catalytic site in SR-39 TK, in which the natural aliphatic-containing amino acids, isoleucine at residue 160 and alanine at residue 168, were replaced by an aromatic-containing amino acid, phenylalanine. Collectively, it is necessary to further investigate the steric environment of the active site in this enzyme.

In contrast to the above strategy to create enzyme libraries for probing of specific substrate, an alternative approach by targeting SR-39 TK with a rational design of the pyrimidine nucleoside analogs or creation of a compound library can also provide useful information. One of the typical methods employed amino-substituted core compounds is achieved *via* amide-bond coupling.[5] Besides, the synthesis of arabinosyl uridine analogs such as FaraU **1**, IaraU **2** and FVaraU **3**, IVaraU **4** have been achieved recently (Fig. 1).[6—9] While arabinosyl thymidine analogs have been demonstrated to be excellent substrates for wild type HSV-1 TK,[3] no relevant report of their corresponding activities against the mutant enzymes has been addressed. Additionally, the mutant enzyme SR-39 TK seemed to be more resistant to the structural variation of the pyrimidine nucleoside as described above.

Combining the above considerations, we wish to undertake a cell survival assay to address whether these arabinosyl uridine analogs exerted a comparable bioactivity profile against the SR-39 TK-transfacted murine prostate cancer cells as that exerted by FEAU.

Experimental

DNA fragment encoding sr-39tk was amplified by PCR using pSP72-sr-39tk plasmid as the template, excised from agarose gel, purified and subcloned onto IRES-IL3 vector using *Nhe*I and *Xho*I as the targeting cloning sites. Inserted plasmids were purified from *E. coli* growing under selection media by using PD100 kit (Geneaid, Taiwan) and the inserted gene sequence was confirmed by sequencing (Pozter Biotech, Taiwan). To prepare plasmid used for transfection experiments, maxiprep prodceure was performed according to manufacturer manual (Qiagen, cat #12145, U.S.A.). Tramp-C1 cells (ATCC, CRL-2730™) was grown in Dulbecco/Vogt modified Eagle's minimal essential medium (DMEM) (GIBCO), supplemented with 10% fetal bovine serum (FBS) (GIBCO), 1% penicillin/streptomycin (GIBCO), 5 μg/ml insulin (Sigma) and 10^{-8} M dihydrotestosterone (DHT) (Sigma) at 37 °C incubator humidified with 5% CO_2. Transfection was performed by using Effectene Transfection Reagent (Qiagen) as followed: A mixture of DNA plasmid (1 μg), buffer EC (150 μl) and the Enhancer (8 μl) were mixed by vortexing for 1 s followed by addition of 1 ml culture media. The mixture was then added to 2×10^5 of Tramp-C1 cells grown on a 10 cm

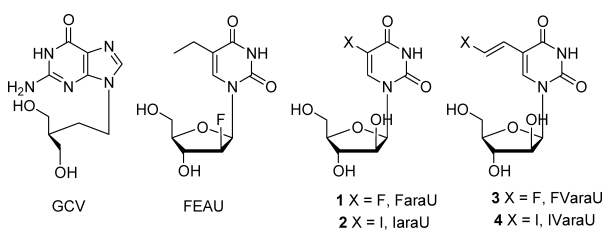

Fig. 1. Potential Gene Probes for HSV-1 TK

* To whom correspondence should be addressed. e-mail: csyu@mx.nthu.edu.tw

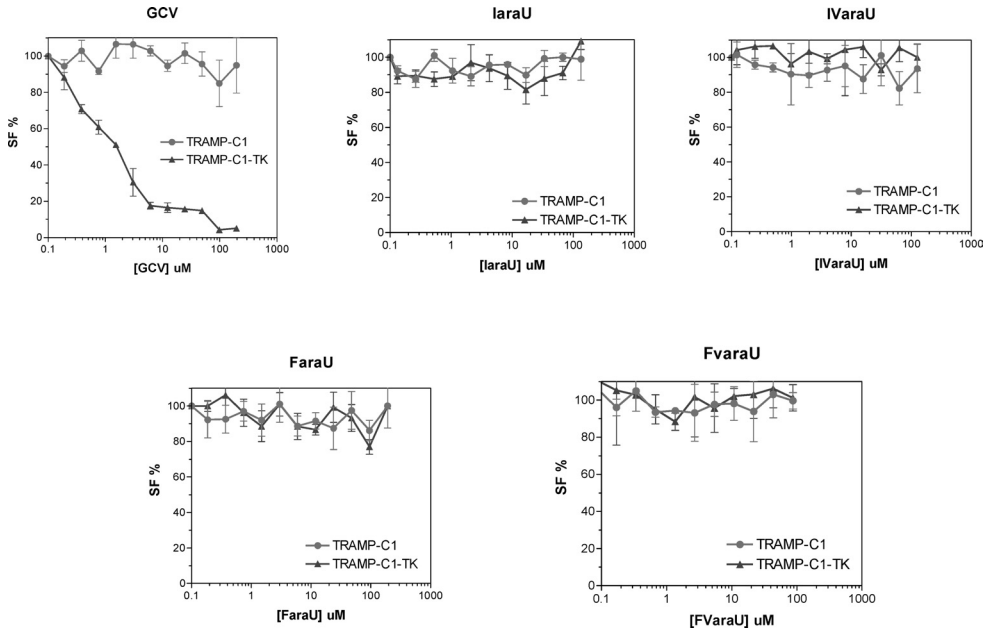

Fig. 2. Comparison of the Cytotoxic Effect of GCV and Arabinosyl Uridine Analogs on TRAMP-C1 and SR-39 TK Transfected TRAMP-C1 Cells (TRAMP-C1-TK)

The cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The surviving fraction (SF) was calculated by the number of viable cells after 5 d incubation following various concentrations of tested agents/the number of viable cells of control treatment. Data and error bars represent the mean±1 S.D. of three repeat experiments with triplicate assay in each experiment. The results of tests of significance are reported as p-values <0.05 and are derived from analysis of Student's t-test using a two tailed distribution. The p-values were calculated using the GraphPad Prism software version 3.03 package (GraphPad Software Inc., San Diego, CA, U.S.A.).

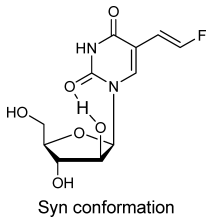

Fig. 3. Proposed *syn* Conformation Derived from Intra-molecular Hydrogen Bonding

tissue culture dish. Two-day post-transfection, cells were treated with G418 (Promega) to select cells which were stably expressed sr-39tk gene. GCV stock solution (10 mg/ml) was made by dissolving in 0.1 N HCl and was further diluted to various concentrations (0—200 μg/ml) in a final volume of 100 μl per treatment on the day of treatment. To assay the efficacy of GCV, 500 Tramp-C1 cells (either with or without sr-39tk overexpression) were incubated with 100 μl of various concentration of GCV in a 96-well plate at 37 °C incubator humidified with 5% CO_2 for 5 d. The cell growth effect was assessed by MTT assay using the wavelength of 570 nm. The 3-D software used in this study is Chem 3D pro 8.0.

Results

As indicated in Fig. 2, GCV exerted a significant cytostatic effect against the cell growth of sr-39tk-transfected Tramp-C1 cells (Tramp-C1-TK) at the concentration of 1 μM (IC_{50}), in contrast to the negligible cytotoxic effect after treating cells with arabinosyl pyrimidine nucleosides. Four arabinosyl nucleosides tested were poorer substrates in our hands regardless of the lack of FEAU in the assay. Gambhir and coworkers reported that radiolabeled [^{3}H]FEAU showed a comparable accumulation as [^{3}H]penciclovir (PCV), an analogue of GCV, in sr-39 tk transfected C-6 rat glioma cell.[4] It was suggested that the intramolecular hydrogen bonding might dictate the directional of the side chain by pushing the

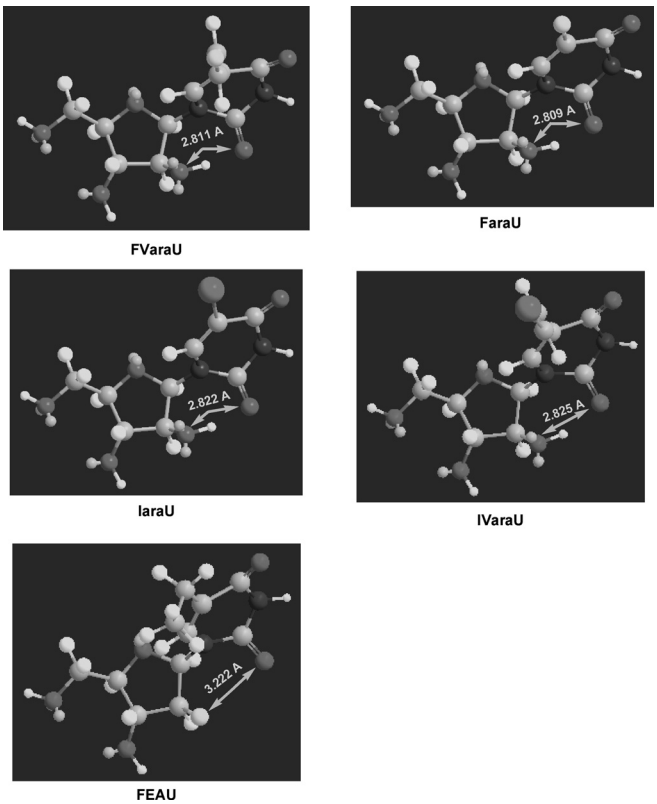

Fig. 4. A 3-D Structural Comparison of FVaraU, FaraU, IaraU, IVaraU and FEAU

bulky aromatic groups towards the active site (Fig. 3). A 3-D molecular modeling coupled with the MM2 program upon energy minimization mode for these compounds had been performed (Fig. 4). All the four arabinosyl uridine analogs

showed a structural similarity in respect of the equivalent distance between the 2′-hydroxyl group of furanosyl moieties and the oxo group at C-2 of uracil base. Contradicting to the relatively loose space between the corresponding 2′-fluoro and 2-oxo, the 2′-hydroxyl group was suggested to involve in hydrogen-bond formation as indicated by an in-between hydrogen atom. Since no such hydrogen bonding is available for fluorine atom in FEAU, the base moiety should adopt the usual *anti*-conformation, thereby allowing a favorable binding. This may partially account for the preservation of activity by FEAU. The preparation and bioassay of a series of compound libraries based on amino arabinosyl uridines are in progress. In brief, the cytotoxicity of four halogen-substituted arabinosyl uridine compounds with potentiality as positron-emitter tagged probes against SR-39 TK transfacted murine prostate cancer cells has been evaluated. A further investigation of the bioactivity against the wild-type HSV TK transfacted cancer cells should be performed to confirm the roles played by this 2′-hydroxyl group.

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