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^{99m}Tc LABELED *N*-ACETYL NEURAMINIC ACID AS A NEW RADIONUCLIDE PROBE FOR TARGETING CANCER: *IN-SILICO* AND *IN-VITRO* STUDY

Ravi Ranjan Kumar [1], Jasmine Sati [1], Monika Chauhan [3], Neelima D. Passi [3], Dewinder K. Dhawan [2] and Vijayta D. Chadha [*1]

Centre for Nuclear Medicine [1], University Institute of Emerging Areas in Science and Technology (UIEAST), Department of Biophysics [2], University Institute of Pharmaceutical Sciences (UIPS) [3], Panjab University, Chandigarh - 160014, Chandigarh, India.

Keywords:

N-acetyl neuraminic acid, *In-vitro* cancer cell, Molecular docking, Radiolabeling, Cytotoxicity, Lectin receptor

Correspondence to Author:
Dr. Vijayta D. Chadha

Center for Nuclear Medicine, (UIEAST), Panjab University, Chandigarh - 160014, Chandigarh, India.

E-mail: vdchadha@pu.ac.in

ABSTRACT: Radioisotopic imaging based on small molecules like carbohydrate has been regarded as a promising candidate for *in-vivo* imaging for cancer diagnosis in recent years. In this study, we report that N-acetyl neuraminic acid (Neu5Ac), a type of sialic acid with 9-carbon amino sugar and low pKa value coordinates with the transition radioactive metal ^{99m}Tc. The radiochemical yield of ^{99m}Tc labeled Neu5Ac was observed to be greater than 90% and was confirmed by Instant Thin Layer Chromatography. Molecular docking studies showed greater affinity of ^{99m}Tc labeled Neu5Ac towards the lectin receptor when compared to cold Neu5Ac. The radiolabeled complex (^{99m}Tc-Neu5Ac) binds specifically to the HT-29 cells and was mildly cytotoxic at a concentration of 402 µM. Cellular internalization of ^{99m}Tc-Neu5Ac was mainly in the cytosolic proteins and free membranes as compared to nuclear fraction and large organelles. In conclusion, Neu5Ac was successfully radiolabeled with ^{99m}Tc, and *in-vitro* binding studies confirmed that our developed radionuclide probe binds selectively to the cancer cells. Further, efficacy of our developed complex may be useful for the *in-vivo* imaging of cancer.

INTRODUCTION: Technetium-^{99m} (^{99m}Tc) is the most prevalent diagnostic radionuclide in nuclear medicine due to its easy availability from commercial generator columns, ideal nuclear properties, and suitable decay characteristics. It emits γ-rays with an energy of 140 keV, which is close to optimal for imaging with commercial gamma cameras. The six-hour half-life is sufficiently long for pharmaceutical preparation and *in-vivo* accumulation in the target [1, 2].

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The ability to include this radionuclide into targeting molecules has been the foremost consideration for developing radiopharmaceuticals. Various ligands have been labeled with ^{99m}Tc by direct method using stannous chloride as the reducing agent.

^{99m}Tc-DTPA (Diethylenetriamine pentaacetic acid) and ^{99m}Tc-MDP (Methylene diphosphonate) are some of the radiopharmaceuticals clinically used to diagnose the disease in nuclear medicine practices [3, 4]. Additionally, some carbohydrates like glucoheptonate and glucuronic acid are carboxylic acid analogs of glucose, which have been coordinated with ^{99m}Tc. These radio complexes formed have been approved for imaging of the brain, kidney, myocardium and tumor necrosis respectively [5, 6].

N-acetyl neuraminic acid (Neu5Ac) is the most predominant type of sialic acid, which consists of 9-carbon carboxylate amino sugar and is involved in numerous fundamental functions of the living cells [7]. Due to its low pKa value (2.2), this carbohydrate is a very good ligand for transition metals due to its ability to donate electrons from the hydroxyl group of Neu5Ac [8, 9]. Overexpression of cell surface sialic acids has been correlated with the metastatic potential of several tumors, and cell surface sialic acid has also been a target for drug delivery [10]. Sialic acid binds to the lectin receptor and is over-expressed in many cancer cells. Numerous studies have also revealed that various cancer cells take up exogenous sialic acid [11, 12, 13, 14]. Till date, the use of sialic acid for tumor imaging has been largely unexplored. In the present study, *N*-acetyl neuraminic acid was radiolabeled with radioactive transition metal ^{99m}Tc by direct labeling method. The radiochemical purity was determined by Instant Thin Layer Chromatography (ITLC). Molecular docking studies were also performed. Cytotoxicity and *in-vitro* binding studies of ^{99m}Tc labeled Neu5Ac were determined in HT-29 human colon cancer cell line.

MATERIALS AND METHODS:

Chemicals: HT-29, a human adherent type colon cancer cell line, was procured from National Centre for Cell Science (NCCS) Pune, India. Dimethylsulfoxide (DMSO), *N*-acetyl neuraminic acid, Roswell Park Memorial Institute medium (RPMI) 1640 media [(supplemented with L-glutamine and sodium bicarbonate ($NaHCO_3$)] and stannous chloride dihydrate ($SnCl_2.2H_2O$) were purchased from Sigma-Aldrich. Pertechnetate ($^{99m}TcO_4^-$) was obtained from the Post Graduate Institute of Medical Education and Research (PGIMER) Chandigarh, India. Instant Thin Layer Chromatography- silica gel (ITLC-SG) strips were purchased from MERCK. Fetal bovine serum (FBS), trypsin-EDTA (10x), antibiotic antimycotic solution (1000X) was purchased from Hi-media.

Radiolabeling and Radiochemical Purity: ^{99m}Tc labelled Neu5Ac was prepared by adding 7.4 Megabecquerel (MBq) (200µCi) of pertechnetate ($^{99m}TcO_4^-$) to a vial containing 50 µg of Neu5Ac (1mg/ml in DDW), 10 µg of stannous chloride dihydrate ($SnCl_2 \cdot 2H_2O$) [1mg $SnCl_2 \cdot 2H_2O$ in 1 ml of 0.01N hydrochloric acid (HCl)] and the pH was adjusted between 6.5 to 7.5 using 0.05M sodium hydroxide (NaOH). The reaction mixture was gently shaken and kept at room temperature for sufficient time to complete the reaction. Percentage labeling of Neu5Ac with ^{99m}Tc was carried out by ascending chromatographic technique.

Briefly, Instant Thin Layer Chromatography-silica gel (ITLC-SG) strips were cut into appropriate width and length (0.5 × 12 cm) and the point of origin and end line (solvent front) were marked from the base [15, 16, 17]. A single spot of preparation was applied on the strip at the point of origin. Strips were then placed in tubes containing absolute (100%) acetone and a mixture of Pyridine: Acetic acid: Water [PAW] (3:5:1.5) as mobile solvents to rule out the amount of free pertechnetate ($^{99m}TcO_4^-$) and reduced/ hydrolyzed (^{99m}Tc-R/H) fraction in the preparation. The strips were left undisturbed in the developing tubes to allow movement of the solvent and then removed after the solvent touched the end line (Solvent front). The strips were air-dried, cut into 1 cm segments, and then counted for activity using a well-type gamma-sensitive probe (ECIL, Hyderabad, India). Finally, the labeled compound was calculated according to the following equations:

% Free $^{99m}TcO_4^-$ = (Radioactivity counts at R_f = 1)/ Total radioactivity counts × 100

ITLC-SG/acetone system

% ^{99m}Tc-R/H = (Radioactivity counts at R_f = 0) / Total radioactivity counts × 100

ITLC-SG/ Mixture of PAW system

Radiochemical purity of ^{99m}Tc-Neu5Ac (%) = 100 – (% Free $^{99m}TcO^-_4$ + % R/H)

Molecular Docking Studies: Three-dimensional (3D) crystal structure of the known potent anticancer drug target lectin was retrieved from the protein data bank (PDB ID: 3WHD; http://www.rcsb.org). Among the various crystal structures available for lectin, 3WHD human lectin receptor with a resolution of 2.29 Å was used in the present study, **Fig. 1**. [18]

Ligand Preparation: One of the important determinants for a successful docking is the structure of the ligand. The 2D structure of the N-acetyl neuraminic acid and its ^{99m}Tc radio labeled

analog was sketched using the ChemDraw ultra 8.0, followed by their conversion into 3D and the chemical structure was saved in the requisite format shown in **Fig. 2a** and **b** [19]. The geometry and energy of these molecules were optimized and minimized using the Merck Molecular Force Field (MMFF) method. Conformer with the lowest energy was selected for docking simulation studies. The comprehensive and integrated graphical user interface program of the Vlife MDS 4.6, *i.e.* ''Bio Predicta module'' was used to prepare, run, and analyze the docking simulations on the HP Pentium IV 2.80 GHz Processor / Microsoft Win XP Home Edition system.

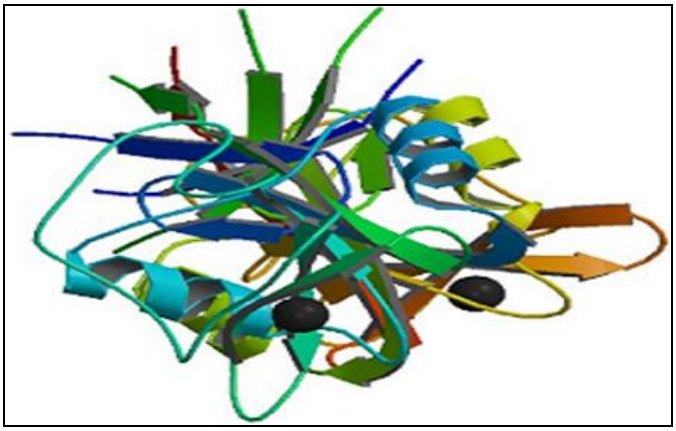

FIG. 1: 3D STRUCTURE OF 3WHD PROTEIN IN NEW CARTOON VIEW

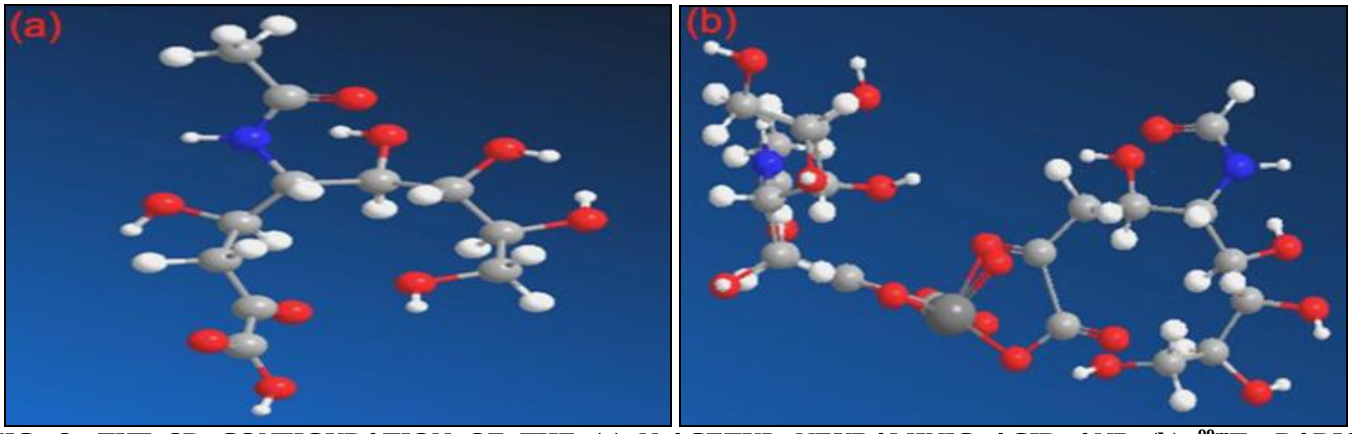

FIG. 2: THE 3D CONFIGURATION OF THE (a) N-ACETYL NEURAMINIC ACID AND (b) ^{99m}Tc RADIO LABELED PROPOSED ANALOGUE WAS SKETCHED USING THE ChemDraw ultra 8.0, FOLLOWED BY THEIR CONVERSION INTO 3D AND CHEMICAL STRUCTURE WAS SAVED IN THE REQUISITE FORMAT.

***In-vitro* Study:** HT-29 cells were used to test the cytotoxicity and internalization of ^{99m}Tc labeled Neu5Ac. HT- 29 cells were grown in RPMI 1640 media supplemented with L-glutamine, sodium bicarbonate, 10% FBS, 100 units/ml of penicillin, 100 µg/ml of streptomycin and 1 mM sodium pyruvate. Cells were grown at 37°C in a humidified incubator with an atmosphere of 5% CO_2 and 95% air. The cell culture flasks were replaced with medium every 3-4 days and passaged when 90-95% confluent.

For subculture of cells, the medium was removed from the flask, and a solution of 0.5% trypsin/0.2% EDTA in 0.85% normal saline (1X) was added. HT-29 cells were incubated at 37°C and 5% CO_2 for 2 min, and an equal volume of medium was added to deactivate the trypsin. The culture was transferred to a 25 ml conical tube and centrifuged at 2000 rpm for 10 min. Cells were re-suspended in medium and seeded into T 175 flasks.

Cytotoxicity by MTT Assay: Cytotoxicity of Neu5Ac was evaluated at 24 h in HT-29 cells using MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) assay [20]. 1×10^4 cells were plated per well in a 96 well plate and were allowed to attach overnight. The next day, fresh media containing different concentrations of ^{99m}Tc-Neu5Ac (8, 16, 32, 40, 80, 100, 160 and 402 µM) were added to the plate in triplicates. After 24 h incubation at 37 °C, the added medium in each well was removed.

Subsequently, 180 µL of RMPI (without FBS) and 20 µL of MTT stock solution (5 mg/ mL in PBS) was added and incubated for 4 h resulting in formation of formazan crystals. The medium containing MTT was then completely removed. Immediately, 200 µL of DMSO was added to each well to dissolve the formazan crystal. Absorbance intensity was measured by an ELISA plate reader (Bio-RAD 680, USA) at 540 nm by a reference

wavelength of 620 nm with linear shaking for 10 sec at 25ºC. Wells containing cells incubated with 10% tritonX-100 were treated as negative control, and those containing assay medium without ^{99m}Tc-Neu5Ac were treated as a positive control. Wells containing assay medium and MTT reagent without cells were used as blank. Cell viabilities were determined by reading the absorbance at 540 nm. The experiment was performed two times in triplicate.

Cell viability (%) = (Absorbance of sample) / (Absorbance of positive control) ×100

Cellular Binding: The cellular uptake studies of ^{99m}Tc-Neu5Ac were performed on HT-29 cell lines. The cells (6×10^5) were seeded in 12-well plates and kept in a humidified 5% CO_2 incubator at 37 ºC overnight. The following day, cells were incubated with the radio-complex (^{99m}Tc-Neu5Ac, 325µM, 4MBq) at 37 °C for 1 h. Cells were simultaneously incubated with $^{99m}TcO_4$ containing the same amount of activity as that of the radio-complex, to account for the uptake of $^{99m}TcO_4^-$ by the cells [21]. Incubation of the samples was terminated by removing the medium and washing the cells twice with ice-cold PBS. After that, the cells were detached by trypsin, and the radioactivity in the cell suspension was quantified. To determine specific versus nonspecific binding, HT-29 cells were seeded into 12-well plates at a density of 6×10^5 cells/well and incubated with unlabeled Neu5Ac at a concentration 500 times higher than the ^{99m}Tc labeled Neu5Ac for 35 min at 37 ºC, and then the radio complex was added to the wells.

Internalization and Sub-Cellular Fraction Binding: To study the binding characteristics of ^{99m}Tc-Neu5Ac, HT-29 cells were plated at a density of 10^6 cells per well in 12-well tissue culture plates and were allowed to attach overnight at 37 °C in a humidified atmosphere with 5% CO_2. The following day, cells were incubated with radio-complex (^{99m}Tc-Neu5Ac, 325µM, 4MBq) for different time intervals up to 4 h. Cellular uptake was stopped at each time interval by removing the medium from the cells and washing cells twice with ice-cold PBS. The cells were subjected to an acid wash (50 mM glycine HCl/100 mM NaCl, pH 2.8) to remove surface-bound radioligands. Immediately, pH was neutralized with cold PBS containing 0.2% bovine serum albumin (BSA) and subsequently, the cells were lysed in 500 µl of lysis buffer (Tris 10 mM, $MgCl_2$ 3 mM, NaCl 10 mM, 0.1% triton X-100 pH 7.5-8.0) at 37 °C. After 30 min of incubation in lysis buffer, the surface-bound (acid-wash) and internalized radioactivity were measured using the gamma well counter [22].

To see the subcellular fraction binding, the cell suspension was removed and centrifuged at 1300 g at 4°C for 5 min to pellet nuclei and cell debris (P1). The supernatant obtained (S1) was further centrifuged at 20,000 g at 4°C for 20 min to obtain free membrane and soluble cytosolic proteins as supernatant (S2) and large intact organelles as a pellet (P2). At different incubation time, the unbound activity (activity outside the cell), cell surface-bound activity and the activity associated to different sub-cellular fractions, (activity in the nucleus (P1), outside the nucleus (S1), free membranes and soluble proteins, *i.e.* cytosol (S2) and large intact organelles (P2) were measured (3 replicates) in a gamma-counter. Total binding represents uptake on the surface membrane and that present inside the cell; internalization represents the activity present inside the cell only.

Statistical Analysis: Experiment studying each factor was repeated three times, and differences in the data were evaluated with one-way analysis of variance (ANOVA) test. Results are reported as mean ± standard deviation (S.D.). The level of significance was set at $p \leq 0.05$. The statistical software package SPSS v 22 for windows were used for the purpose.

RESULTS AND DISCUSSION:

Radiolabeling and Radiochemical Purity: The radiochemical purity of the ^{99m}Tc-Neu5Ac complexes was determined by ascending instant Thin Layer Chromatography. In ITLC (SG) using acetone as the solvent, free pertechnetate moves with the solvent front (R_f =1), while ^{99m}Tc-Neu5Ac complex and reduced/hydrolyzed technetium (^{99m}Tc-R/H) stayed at the bottom (origin).

^{99m}Tc-R/H was assessed by using the mixture Pyridine: Acetic acid: Water (3:5:1.5) as the mobile phase where ^{99m}Tc-R/H remains at the origin (R_f= 0) while other species migrated with the solvent front (R_f=0.8) **Table 1**. The radiochemical purity was determined by subtracting the sum of the

percent of colloid and free $^{99m}TcO^{-}_{4}$ from 100%. The maximum radiochemical yield of ^{99m}Tc labeled Neu5Ac was 93.4 ± 0.87 %. The ITLC radio chromatogram is presented in **Fig. 3**.

TABLE 1: RETENTION VALUES (R_f) OF ^{99m}Tc (PERTECHNETATE), REDUCED/HYDROLYZED (^{99m}Tc -R/H) AND ^{99m}Tc-Neu5Ac WAS DETERMINED USING ASCENDING ITLC (SG) IN TWO DIFFERENT SOLVENT SYSTEMS

Solvents Systems	R_f value		
	Free ^{99m}Tc	^{99m}Tc-R/H	^{99m}Tc-Neu5Ac
Acetone	1	0.00	0.00
Pyridine: Acetic acid: Water (3:5:1.5)	0.9-1	0.0-0.2	0.8-0.9

Data are represented as mean values ± standard deviation, n = 4.

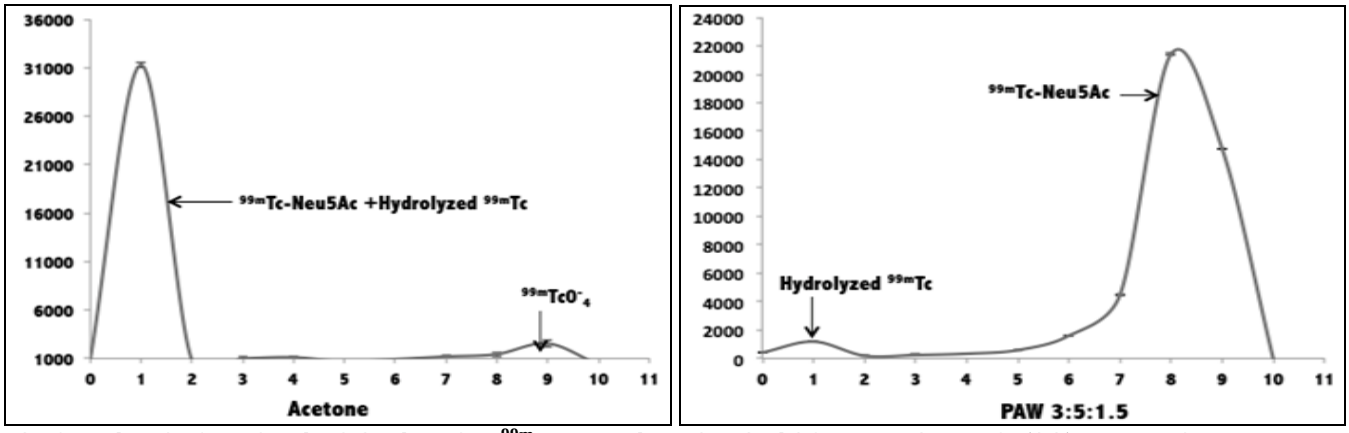

FIG. 3: RADIO CHROMATOGRAM OF ^{99m}Tc-Neu5Ac USING ASCENDING ITLC (SG) IN TWO DIFFERENT SOLVENT SYSTEMS. Data are represented as mean values ± standard deviation, n = 4.

Docking Studies: The molecular docking studies showed that the most stable *N*-acetyl neuraminic acid conformer, *i.e*. ligand pose 1 bind to the active site of residues of the lectin with greater affinity represented by a low docking score of -41.60. The docked complex was further analyzed for the active involvement of molecular interaction with 3WHD, indicating the hydrogen and hydrophobic interaction, as shown in **Fig. 3**. This was noticed to contribute one conventional hydrogen bond between the oxygen with LYS68A by 2.98 Å distance. Further, it was found to form one charge interaction between oxygen and LYSC Å with a bond distance of 4.53 Å. Apart from these interactions, carbon of the ligand was found to show hydrophobic interactions with LYSC, LEU64A, ALA62A, LEUC and PRO85A with a bond distance of 4.12 Å, 4.22 Å, 3.72 Å, 4.75 Å and 4.89 Å respectively as shown in **Fig. 4A** & **B**.

Similarly, docking results for ^{99m}Tc labeled analog (ligand pose 8) complex with 3WHD showed a good affinity and affording D-Score -91.69. The favorable D-Score could be attributed to its strong hydrogen and hydrophobic interaction with amino acids of the active site of the receptor.

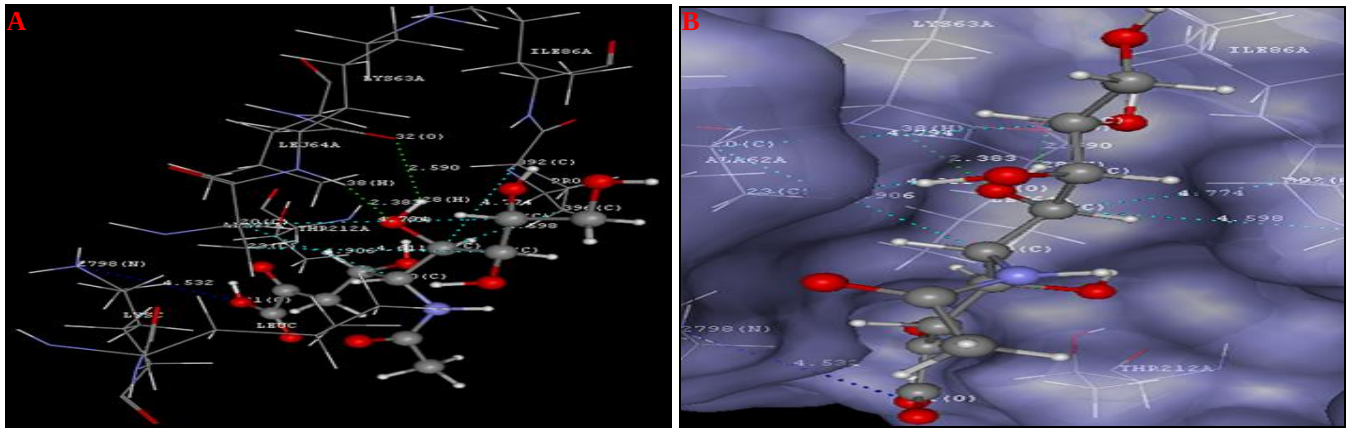

FIG. 4: (A) THREE-DIMENSIONAL STRUCTURAL REPRESENTATION OF MOLECULE IN THE BINDING DOMAIN OF 3WHD. (B) INTERACTIVE FORCES INVOLVED BETWEEN LIGAND AND AMINO ACIDS IN THE BINDING POCKET

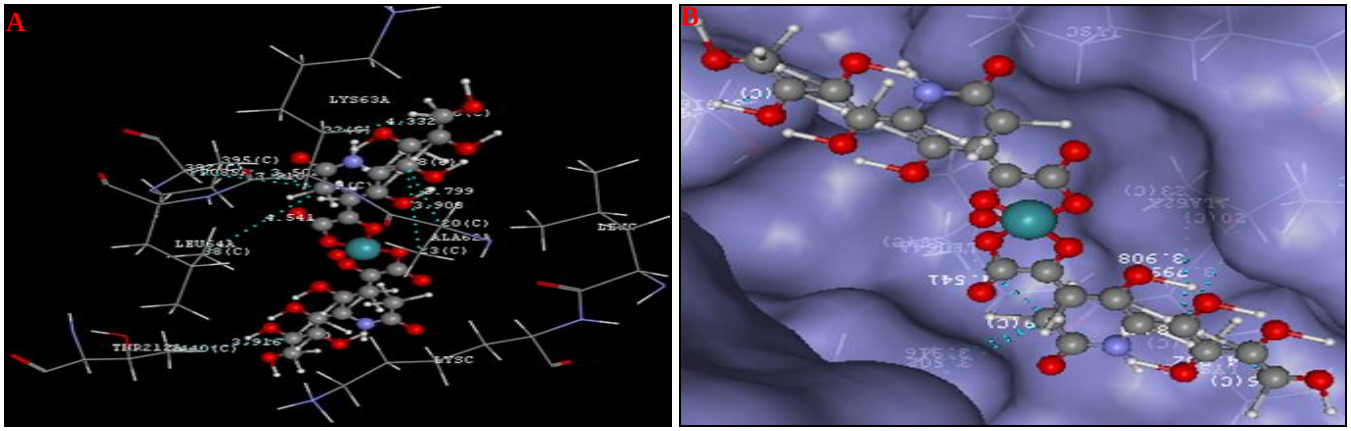

FIG. 5: (A) THREE-DIMENSIONAL STRUCTURAL REPRESENTATION OF MOLECULE IN THE BINDING DOMAIN OF 3WHD. (B) INTERACTIVE FORCES INVOLVED BETWEEN LIGAND AND AMINO ACIDS IN THE BINDING POCKET

Three oxygen atoms were involved in hydrogen interaction with LYS63A with a bond length of 2.07 Å, 2.59 Å, and 2.45 Å respectively. Further, one oxygen found with Pro 210A by a distance of 1.58 Å. At the same time, carbon atoms showed a greater contribution in hydrophobic interactions with ALA624A, CYS84A, PRO85A, THR212A and LEU64A with the bond length of 3.95 Å, 4.76 Å, 3.79 Å, 3.53 Å and 3.20 Å respectively as shown in **Fig. 5A** & **5B**. It has been observed from the docking score that the ^{99m}Tc labeled analog of N-acetyl neuraminic acid binds to the lectin receptor with greater affinity and requires almost half of the binding energy as required by the N-acetyl neuraminic acid. Several reports have also demonstrated higher affinity of the radiolabeled complex with the receptor [23, 24].

MTT Assay: MTT is a water-soluble tetrazolium salt, which is transformed to an insoluble purple formazan by cleavage of the tetrazolium ring by succinate dehydrogenase within the mitochondria. The formazan product formed is impermeable to the cell membrane and accumulates in the healthy cell and thus is a measure of cell viability. HT-29 cell lines were taken to screen for the *in-vitro* cytotoxic activity of ^{99m}Tc-Neu5Ac for a range of different concentrations, as displayed in **Fig. 6**. There was no change in cell viability in the concentration range from 8-100 µM of ^{99m} Tc-Neu5Ac. At 8 µM concentrations, the cell viability was observed to be 97.1 % ± 0.82. However, at concentration of 160 and 402 µM, the cell viabilities observed were 88% ± 0.11 and 86% ± 0.62 respectively. There was a significant decrease in cell viability at a concentration of 160 and 402 µM ($p<0.05$) when compared to the control cells. Similar to our finding, no detrimental effects on cell viability were observed at doses up to 1 mM for FITC labeled Neu5Ac [14].

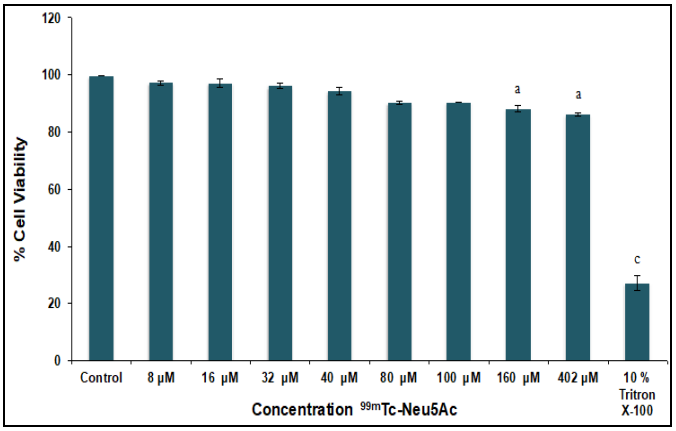

FIG. 6: CELL VIABILITY OF ^{99m}Tc-Neu5Ac AGAINST HT-29 HUMAN COLON CANCER CELL LINE. Data are represented as mean ± standard deviation, n = 3. $^{a}p\leq0.05$, $^{b}p\leq0.01$, $^{c}p\leq0.001$ are calculated by Least Significant Difference using one way ANOVA when values are compared with the control group.

***In-vitro* Cellular Binding:** The cell-associated radioactivity of ^{99m}Tc-Neu5Ac was higher in HT-29 cancer cells when compared to the ^{99m}Tc alone as depicted in **Fig. 7A**. This shows that the radiolabeled complex binds specifically to the HT-29 cells as compared to ^{99m}Tc alone. The *in-vitro* cell-binding competitive assay was performed by addition of 500-fold excess of cold Neu5Ac as a competitor with ^{99m}Tc-Neu5Ac. The saturation of the binding sites on HT-29 cells with Neu5Ac resulted in a decrease in radioactivity when compared to the unblocked sites. This demonstrated that the binding was through Neu5Ac as shown in **Fig. 7B**.

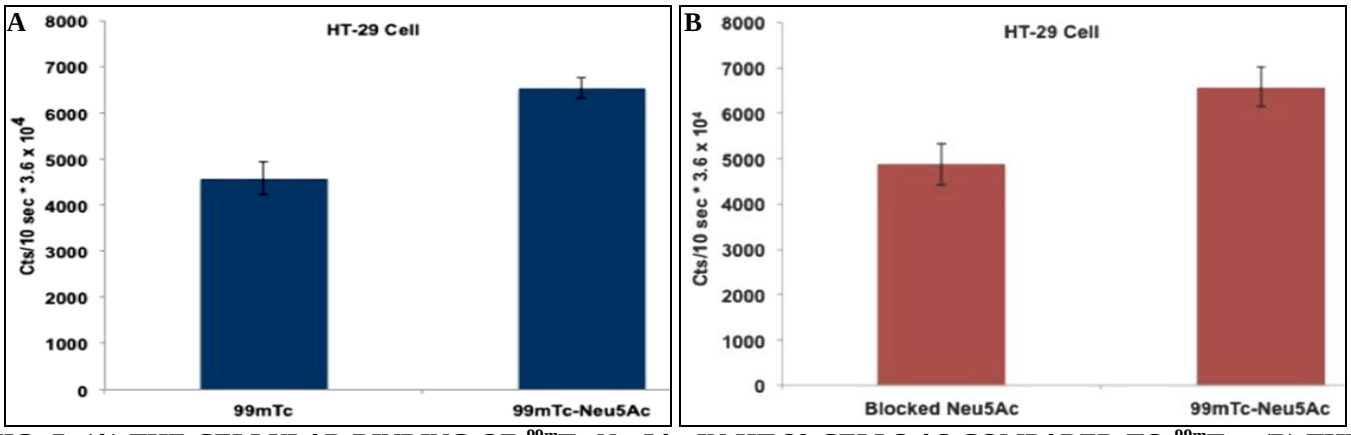

FIG. 7: (A) THE CELLULAR BINDING OF ^{99m}Tc-Neu5Ac IN HT-29 CELLS AS COMPARED TO ^{99m}Tc. (B) THE CELLULAR BINDING BY PRE-SATURATION OF THE HT-29 CELLS BY USING 500-FOLD EXCESS OF COLD Neu5Ac BEFORE ADDING THE ^{99m}Tc-Neu5Ac. Data are represented as mean values ± standard deviation, n = 5. Statistical significance was considered at $p \leq 0.05$.

Internalization and Sub-Cellular Fraction Binding Study: The *in-vitro* total binding and internalization experiment was performed to determine the rate and fraction of internalization of ^{99m}Tc-Neu5Ac in HT-29 human colon cancer cells. The percentage of total binding (Activity present on cell surface +activity inside the cell) of Neu5Ac after 15, 30, 60, 120 and 240 min was observed to be 8.0 ± 0.2%, 7.1 ± 0.6%, 6.6 ± 0.25%, 6.8 ± 0.75% and 6.1 ± 0.65% of the total radioactivity in the cells. The results of *in-vitro* time dependency internalization and total binding of ^{99m}Tc-Neu5Ac are shown in **Fig. 8A**. Maximum internalization was observed at 2 h and 4 h *i.e.* 2.65 ± 0.6% and 2.1 ± 0.13% respectively. **Fig. 8B** shows the binding of ^{99m}Tc-Neu5Ac in different cellular fractions of HT-29 cells following incubation at 37 °C for 2 and 4 h. These time frames were selected since maximum internalization has been found to occur in this period.

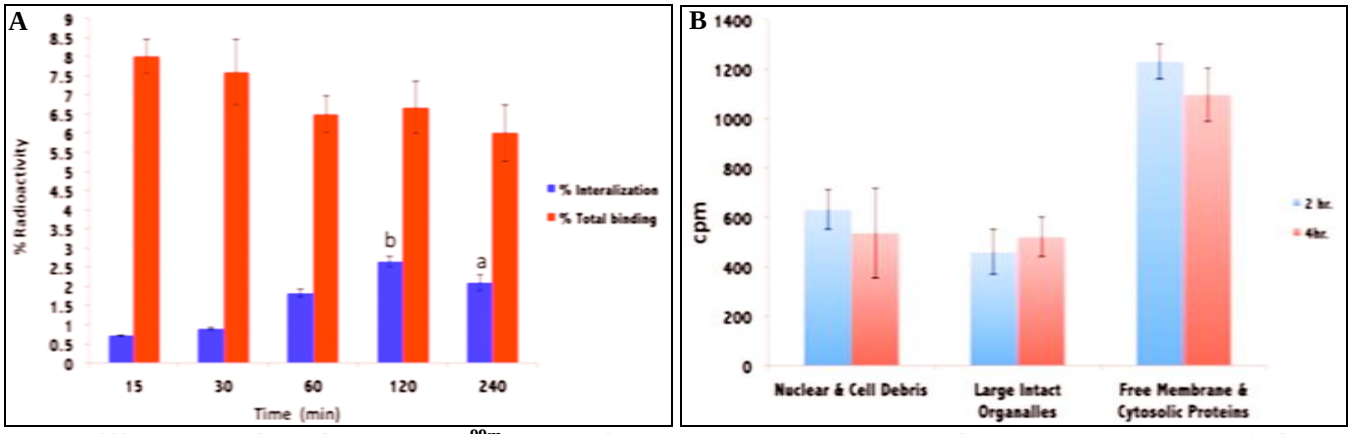

FIG. 8: (A) INTERNALIZATION OF ^{99m}Tc-Neu5Ac IN THE HT-29 CELLS AT DIFFERENT TIMES AFTER INCUBATION AT 37 °C. (B) BINDING OF ^{99m}Tc-Neu5Ac TO DIFFERENT CELLULAR FRACTIONS OF HT-29 CELLS. Data represent mean values ± standard deviation, n = 5. The lowercase alphabet a, b & c represented the statistical difference ($^{a}p \leq 0.05$, $^{b}p \leq 0.01$, $^{c}p \leq 0.001$) when % internalization was compared with total binding.

The present study was performed to determine the cellular fraction (s) enriched in binding sites for the radio-complex.

Radioactivity associated with each fraction represented the counts per minute of total internalized radio-complex bound to the respective cell fraction. These results suggest that the percentage internalized ^{99m}Tc-Neu5Ac was bound more to the soluble cytosolic proteins and free membranes, whereas binding to the nuclear and large cellular components was much less.

CONCLUSION: The developed radio complex ^{99m}Tc-Neu5Ac showed a high labeling efficiency of more than 90%. *In-silico* screening indicated that ^{99m}Tc labeled analogs of *N*-acetyl neuraminic acid bind the lectin receptor with greater affinity. *In-*

vitro studies showed that this radio complex binds specifically to HT-29 cells and are internalized in cytosolic proteins and free membranes. Therefore, Neu5Ac could be a novel molecule for targeting tumor by using it as a *in-vivo* radionuclide imaging probe. Our department is continuously making efforts towards it.

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CONFLICTS OF INTEREST: The authors declare no conflict of interest.

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