Original Article

# Chemoinformatic Study and *In vitro* Bioevaluation of Tc-99m-Labeled N-Acetyl Neuraminic Acid in C6 Glioma Cells: Potential Role as a Radionuclide Imaging Probe

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## Abstract

**Background:** To date, the use of sialic acid that are reported to be elevated during malignancy has been largely unexplored for tumor imaging. The purpose of the present study was to study the modeled stable conformers of n-acetyl neuraminic acid (Neu5Ac) and its radiolabeled conjugate (Tc-99m-Neu5Ac) through computational chemistry approach and its *in-vitro* bioevaluation in rat C6 cell lines. **Materials and Methods:** The Neu5Ac was radiolabeled with Tc-99m using stannous reduction method and the radiochemical purity of Tc-99m-Neu5Ac was determined by instant thin layer chromatography.A Cheminformatic study of Tc-99m-Neu5Ac was performed by using Marvin application of ChemAxon. Glioma cancer cells were taken to evaluate the cytotoxicity and binding efficacy of Tc-99m-Neu5Ac. **Results:** Cheminformatic studies exhibited that the most stable conformer of Tc-99m-Neu5Ac is 15 kcal/mol more stable energetically over least stable conformer. The radiochemical yield of Tc-99m labeled Neu5Ac was observed to be greater than 90%. Further, the radiolabeled complex (Tc-99m-Neu5Ac)exhibited specificity for C6 glioma with time and concentration dependent cytotoxicity. **Conclusion:** In conclusion, Tc-99m-Neu5Ac has the potential to be exploited as an in-vivo radionuclide probe for tumor imaging.

**Keywords:** *3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide assay, cheminformatic studies, in vitro bioevaluation, rat C6 cell lines, Tc-99m-Neu5Ac*

## Introduction

Numerous investigations on altered characteristics of malignant cells have shown that altered cell surface is the hallmark of malignant cells.[1,2] In the same context, altered glycosylation of glycoconjugates is one of the important molecular changes that accompany malignant transformation.[3,4] Sialic acid, the end moieties of the carbohydrate chain of glycoconjugates, is reported to be elevated during malignancy.[5,6] The large number of clinical studies to date shows that the intensity of sialylated antigens expression on cancer cells significantly correlates with the prognosis of patients. A statistically significant correlation between the postoperative patient prognosis and sialylated antigen expression has been reported for colon and stomach cancers, while its correlation with $SL^{e}/^{x}$ expression has been reported for lung, breast, prostate, stomach, colon, and urinary bladder cancers.[7]

To date, the use of sialic acid for tumor imaging has been largely unexplored. Because of the clear association between sialylated antigen overexpression and tumor aggressiveness, the imaging of tumor sialylation using radionuclide-imaging approach can offer a concomitant study to imaging not only to diagnose cancer but also to assess patient's prognosis.[8] In the same regard, the earlier study from our lab have reported the initial Characterization of Tc-99m Labeled N-acetyl neuraminic acid (Neu5Ac) for its Application in *in vivo* Imaging of Cancer.[9,10] Therefore, the present study is conceived with an aim to study the modeled stable conformers of Neu5Ac and its radiolabeled conjugate (Tc-99m-Neu5Ac) through computational chemistry approach. Molecular descriptors provide a great deal of knowledge[11,12] regarding the potential candidature of such radiolabeled conjugates and have now become some of the most important variables used in molecular modeling, and



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consequently, managed by statistics, chemometrics, and chemoinformatics.[13] The manipulation and analysis of chemical structural information of the designed novel radiolabeled conjugate Tc-99m-Neu5Ac can be efficiently achieved through the use of molecular descriptors, for example, log *P*, molar refractivity, polarizability, etc., Comparison of these values with those of unlabeled Neu5Ac molecule can provide a better insight and understanding of the structural details of the radiolabeled Neu5Ac. Further, the specificity of the radio complex Tc-99m-Neu5Ac has been assessed by determining its *in vitro* binding in rat C6 cell lines.

## Materials and Methods

### Chemicals

C6 (Glioma) adherent type cancer cell lines were procured from National Centre for Cell Science (NCCS) Pune, India 411007. Dimethylsulfoxide (DMSO), Neu5Ac, Dulbecco's Modified Eagle Medium (DMEM), and stannous chloride dihydrate ($SnCl_2.2H_2O$), Neu5Ac (sialic acid), and sodium perrhenate ($NaReO_4$) were purchased from Sigma-Aldrich (St. Louis, Missouri, United States). Instant thin layer chromatography (ITLC)-silica gel strips were purchased from MERCK USA. Fetal bovine serum (FBS), trypsin-EDTA (10x), antibiotic antimycotic solution (1000x) was purchased from HIMEDIA. Pertechnetate ($Tc\text{-}99mO_4^-$) was obtained from Post Graduate Institute of Medical Education and Research, Chandigarh, India.

### Cheminformatic studies

Cheminformatics has been used to have a deep insight into the structural and various other important chemical parameters of the Tc-99m-Neu5Ac molecular complexes using Marvin application of ChemAxon.[14] Cheminformatics, an amalgam of information and computers, are useful in addressing the chemical behavior of such complexes *in vivo*.

Owing to the vitality of Neu5Ac, approach is to observe the change in the parameters such as solvent accessible surface area (ASA), polar surface area (PSA), charge distribution, log P, and hydrogen bond donor/acceptor sites. Calculations of these parameters correspond to the configuration of the most stable energy conformer.

### Radiosynthesis of Tc-99m-Neu5Ac

Radiosynthesis of Tc-99m-Neu5Ac was carried out by stannous reduction method as reported previously.[9] Briefly, Tc-99m-Neu5Ac was prepared by adding 7.4 Megabequerel (MBq) (200 μCi) of pertechnetate ($Tc\text{-}99mO_4^-$) to a vial containing 50 μg of Neu5Ac (1 mg/ml in DDW) mixture, 10 μg (1 mg $SnCl_2{\cdot}2H_2O$ in 1 ml of 0.01N hydrochloric acid [HCl]) of stannous chloride dihydrate ($SnCl_2{\cdot}2H_2O$) was added, and the pH was adjusted between 6.5 and 7.5 with 0.05M sodium hydroxide (NaOH). Percentage labeling of Neu5Ac with Tc-99m was carried out by ITLC.[9]

### Fourier-transform infrared spectroscopy

A common practice to characterize Tc-99m compounds is with that of the surrogate rhenium complexes prepared at the macroscopic scale, since technetium and rhenium, transition metals of group VIIB of the periodic table share similar coordination chemistry. Therefore, coordination of Re with Neu5Ac was performed with Neu5Ac: $NaReO_4$ (molar ratio = 10:1), 0.5 mL of 1 mg/mL Neu5Ac solution, 50 μL of 1 mg/mL $NaReO_4$ solution, and 50 μL of 1 mg/mL $SnCl_2 2H_2O$ solution in 0.1 M HCl added in clean glass vial. The pH was adjusted 6.5–7.5 with 0.05 M sodium hydroxide (NaOH). The reaction was incubated at 25°C for 20 min. The reaction resultant was lyophilized and the freeze dried powder was employed to collect infrared spectra(4000–500 $cm^{-1}$ Thermo Scientific Nicolet iS50 Fourier-transform infrared spectroscopy [FTIR] spectrophotometer, SAIF/CIL, Panjab University Chandigarh). Similarly, powder forms of cold rehenium and Neu5Ac FTIR spectra were taken for analyzing the shifting of hydroxy functional group present in Neu5Ac when compared it with powder form of reaction mixture.

### Cell culture

C6 rat glioma cancer cells were taken to test the internalization and cytotoxic studies of developed Tc-99m-Neu5Ac radio complex.

#### *Subculture of cells*

Medium was removed from the flask and a solution of 0.5% trypsin/0.2% EDTA in 0.85% normal saline (1X) was added. C6 rat cancer 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. Cultures were replaced with medium every 3–4 days and passaged again when 90%–95% confluence has been achieved.[15]

#### *Preparation of cells from a monolayer culture for counting*

Cells were first observed to evaluate their morphology and their degree of confluence. Briefly, medium was removed from the flask, and 2.5 ml phosphate buffer saline (PBS) was added to wash the cells. Following washing, 4.5 ml trypsin-EDTA was added to the flask and then placed in an incubator (37°C, 5% $CO_2$) for 2–3 min. The cells were then observed under the microscope for their degree of detachment. Fresh medium was added to the fully detached cells and centrifuged at 2000 rpm for 10 min. The Pellet was re-suspended in fresh medium and a part of cell suspension was mixed with 0.3 ml PBS and 0.5 ml 0.4% trypan blue. The cells were observed using hemocytometer slide for cell density.

### *Intracellular uptake of Tc-99m-Neu5Ac molecular complex*

The cellular uptake of Tc-99m-Neu5Ac was performed on C6 cancer cell lines. The cells ($6 \times 10^5$) were seeded in 12-well plates in humidified 5% $CO_2$ at 37°C overnight. The following day, cells were incubated with the radiocomplex (Tc-99m-Neu5Ac, 325 µM,4 MBq) at 37°C for 1 h. Cells were simultaneously incubated with same amount of Tc-99m$O_4^-$ activity as that of the radiocomplex. Incubation of the samples was terminated by washing the cells twice with ice-cold PBS. After trypsin-induced detachment, the radioactivity in the cell suspension was quantified. To determine specific versus nonspecific binding, C6 cells were seeded into 12-well plates at a density of $6 \times 10^5$ cells/well and incubated with unlabeled Neu5Ac at a concentration 100 times higher than the Tc-99m labeled Neu5Ac for 30 min at 37°C, and then the radio complex was added to the wells. The sample radioactivity was counted in well-type gamma-sensitive probe (ECIL, Hyderabad, India).

### *Cytotoxicity by 3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenylte trazoliumbromide assay*

Cytotoxicity of Neu5Ac was evaluated in C6 ($1 \times 10^4$) cells using3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), by modified method of Mosmann, 1983.[16] The cytotoxicity was evaluated at different time intervals, namely, 24 h, 48 h, and 72 h. Briefly, DMEM (with 10% FBS, 1% penicillin/streptomycin) was used to grow the cells overnight. After removing the original medium in each well, 100 µl without serum DMEM containing added to well. Finally, 100 µl of Neu5Ac were added to the designated wells from the stock solution. The final concentration of these Neu5Ac on plate well of C6 cells ranged from 0.8 mM to 8 mM in 200 µl. After different time incubations at 37°C, the added medium in each well was removed and 180 µl DMEM (without FBS) and 20 µl of MTT stock solution (5 mg/ml in PBS) were added the resultant formazan crystals were formed after an incubation of 4 h. The medium containing MTT was then completely removed. Immediately, 200 µl of DMSO was added to each well to dissolve formazan crystal. To determine the cell viability, absorbance was measured by an ELISA plate reader (Bio-RAD 680, USA) at 540 nm with a reference wavelength of 620 nm. Six replicate wells were run for each concentration.

## Results and Discussion

### Cheminformatic studies

Structural studies are very helpful in providing an insight into the possible *in vivo* interactions of such complexes. Low energy conformers have been generated, and the lowest or the most stable energy conformer has been selected for further calculations. Most stable conformer are 15 kcal/mol more stable energetically over least stable conformer [Figure 1]. It is clear from the graphics that the minimum energy of the most stable conformer can be attributed to the extended backbone geometry and transposition of the hydroxyl groups present in the complex. Molecular dynamic studies performed for 1000 fs for the most stable energy conformer did not show any marked change in conformation or energy during simulation.

The solvent accessible molecular surface area (ASA) gives a measure of the contact area of a molecule with the surface and is useful in calculating transfer free energy to move a molecule from polar to a nonpolar environment. ASA has been calculated using the radius of the solvent (1.4 Å for the water molecule) and has been found to be 542.19 Å$^2$, in close proximity to that of the selected crystallographic structure of unlabelled Neu5Ac molecule (485 ± 5 Å$^2$; PDB: 3B50, 2ZOE).[12,13] This implies that interaction of the solvent molecules with the Tc-99m–Neu5Ac complex will not be affected, and thus, the radiolabeled molecule will be recognized as a "self" molecule. Furthermore, the PSA is a potential parameter, which is associated with the passive molecular transport through membranes and therefore, allows the prediction of transporting properties of drugs. PSA of the complex is found to be 199.9 Å$^2$ and is in good correlation with the PSA of the sialic acid (184.6 Å$^2$).

Negative charge of multiple hydroxyl moieties plays a crucial role in the case of sialic acid molecule.[17] Hence, calculations on charge density have been performed and are shown in Figure 2a. It is clearly apparent from the graphics that the negative charge density of terminal carboxylic oxygen and carbonyl oxygen remains same even after labeling with Tc-99m.

LogP value is of great importance in QSAR analysis and rational drug design. XlogP or atom-additive logP value of Tc-99m–Neu5Ac complex has been calculated under resonating conditions and is −4.94 [Figure 2b]. This value is in well accordance with the XlogP value of sialic acid (−4.46) which shows that the complex interaction of

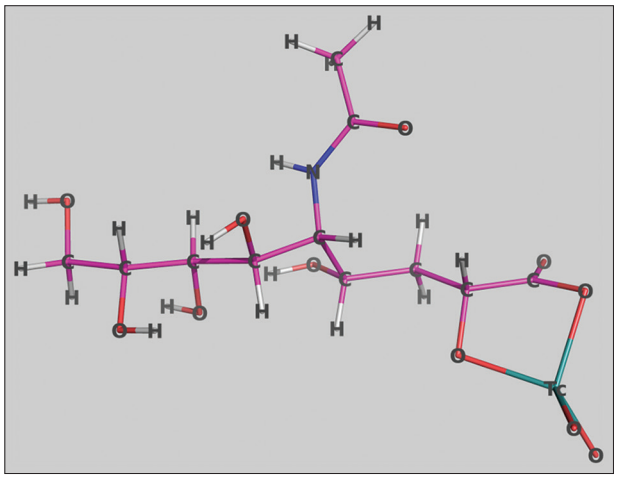

**Figure 1: Most stable energy conformer of Tc-99m-Neu5Ac molecular complexes**

sialic acid with various molecules is due to the presence of multiple hydroxyl groups that are potential hydrogen donor/acceptor moieties. Number of hydrogen bond donor/acceptor sites has been calculated to be 6/9, respectively, for Tc-99m radiolabeled molecular complex [Figure 2c] and are in well agreement with that of the sialic acid molecule, i.e., 7 hydrogen bond donors/10 hydrogen bond acceptors.[13] This further suggests that negligible change in the number of H bond D/A sites will not lead to unwanted side interactions of pertechnetate ion with other molecules *in vivo*. These parametric studies emphasize that complexation of Neu5Ac with Tc-99m molecule will not affect the interaction of hydroxyl groups or N-acetyl moiety, which are necessary for functioning of Neu5Ac molecule. Therefore, radiolabeling of Neu5Ac will not affect its receptor binding ability.

## Radiosynthesis of Tc-99m-Neu5Ac

The radiochemical yield of Tc-99m-Neu5Ac was determined by ITLC using acetone as mobile phase for the assessment of free pertechnetate fraction. Free pertechnetate moved to solvent front in acetone with a retardation factor (Rf) value of 1, while bound and hydrolyzed remained at the origin (Rf = 0). The mixture of pyridine: acetic acid water (3:5:1.5 v/v) was used as a mobile phase to assess the hydrolyzed fraction. In this mixture, bound and free pertechnetate moved to solvent front Rf value of 0.8–1 while hydrolyzed fraction remained at the application point. Percentage radiochemical yield of Tc-99m-Neu5Ac was found to be more than 90%.[18]

## Fourier-transform infrared spectroscopy

Recorded spectra are presented in Figure 3a. The hydroxyl group of Neu5Ac shows at 3237 $cm^{-1}$ whereas the rhenium coordinated Neu5Ac has shifted at 3290 $cm^{-1}$. Poly hydroxyl group present in Neu5Ac showed strong and broadband at region of 3237 $cm^{-1}$ whereas complex showed at 3290 $cm^{-1}$. This suggests that polyhyroxyl oxygen and carboxyl oxygen present in Neu5Ac ligand might have chelated with oxo core of (Re/Tc). Studies also revealed that sialoconjugates plays a role in stabilizing metal ion through the oxygen donor atom derived from the alcohol and carboxylato groups.[19] Therefore, various predictive structures have been drawn on this assumption shown in Figure 3b and also reported earlier from our lab.[9] However, the exact structure of Tc-99m-Neu5Ac needs further exploration through NMR and crystallography.

## Cell culture studies

### *Cellular uptake of Tc-99m-Neu5Ac*

To compare the cellular uptake of the radio labeled complex Tc-99m-Neu5Ac relative to pertechnetate ion, quantitative and qualitative studies were performed. The results of the quantitative assay as shown in Figure 4a confirm that at 1 h, the activity is 1.6 times higher per 3.6 × $10^4$ cells suggesting the increased cellular concentrations of radio labeled complex than those of free pertechnetate ions. These findings can be owed to the phenomenon of

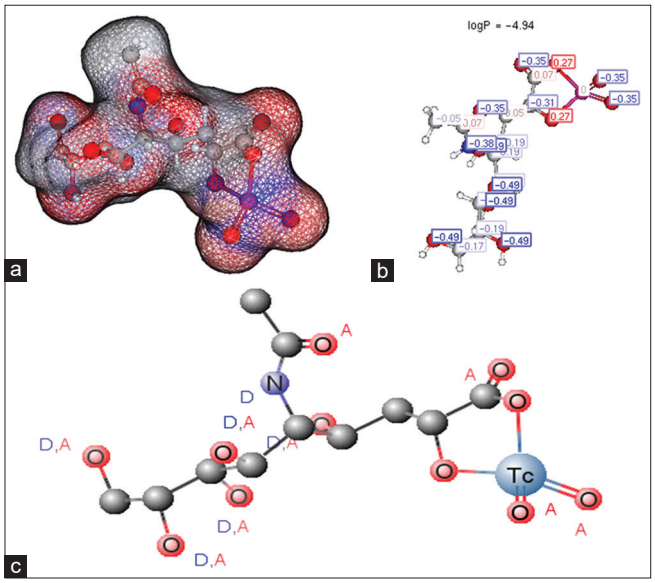


**Figure 2: Graphical representation of major physicochemical properties of radio labeled Tc-99m-Neu5Ac molecular complex showing (a) surface charge density, (b) lipophilic (logP) values, and (c) potential hydrogen donor/acceptor moieties**

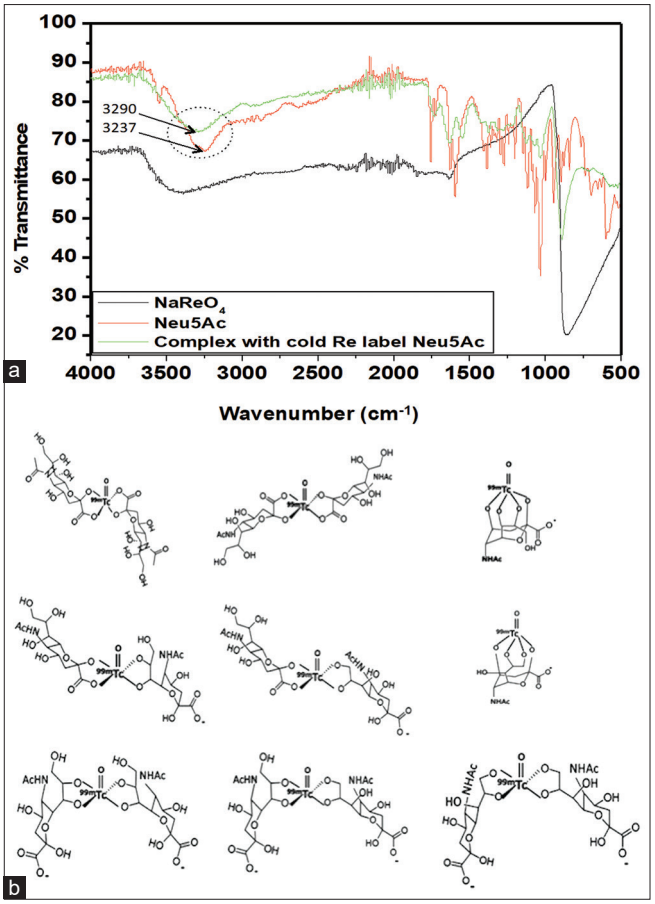


**Figure 3: (a). Fourier-transform infrared spectroscopy spectra recorded immediately of lyophilization of reaction mixture. (b). Various possible putative structure of oxo Tc-99m-Neu5Ac**

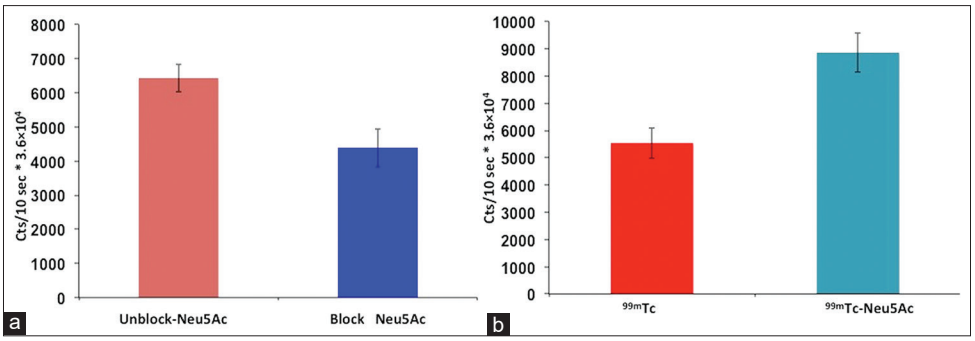


**Figure 4: (a) The cellular binding by pre-saturation of the C6 cells using 500-fold excess of cold Neu5Ac before adding the Tc-99m-Neu5Ac. (b) The cellular binding of Tc-99m-Neu5Ac in C6 cells as compared to Tc-99m. Data are represented as mean values ± standard deviation, *n* = 5. Statistical significance was considered at *P* ≤ 0.05**

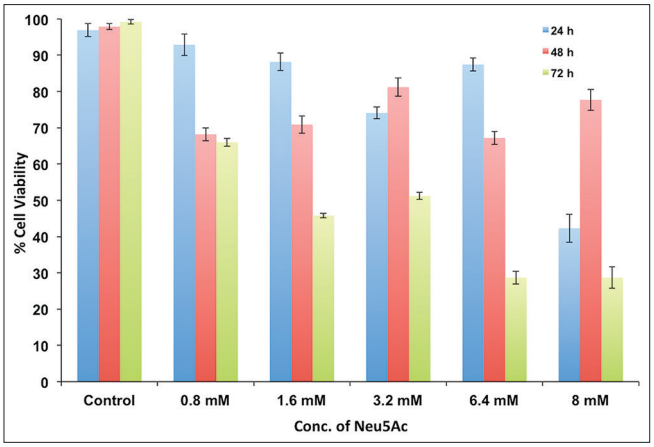


**Figure 5: Cytotoxicity of Neu5Ac against C6 rat cancer cell line. Cell viability 3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide assays were performed in the C6 cell line of rat glioma treated with Neu5Ac at 24 h, 48 h, and at 72 h. Data represent mean values ± standard deviation, *n* = 6; for each given concentration. Statistical significance was considered at *P* ≤ 0.05**

hypersialylation in cancer cells.[8] Further, receptor blocking was done by preincubating C6 cancer cells with 100-times higher concentration of cold Neu5Ac, 30 min before the incubation with Tc-99m-Neu5Ac that reduced the cellular uptake by 1.4 times in blocked cells 1 h postincubation. This is due to the selective binding of the cold Neu5Ac molecules with sialic acid receptors (siglecs) present in the cancer cells [Figure 4b]. This further evidences the selective uptake of Tc-99m-Neu5Ac inside the cells and does not pertain to the surface radioactive contamination/ background.

### *Cytotoxicity of Neu5Ac in C6 cells*

Cell viability and cytotoxicity assays are considered to be vital and are widely used to screen for cytotoxicity of trial drugs. The cytotoxicity profiles of Tc-99m-Neu5Ac on C6 rat glioma cancer cells after treating for 24 h, 48 h, and 72 h are shown in Figure 5. It is clear from the results that after 24 h of treatment, the novel compound did not show growth inhibition of C6 rat glioma cancer cells below a concentration of 6.4 mM. However, cell count decreased as a function of time after 72 h of treatment with Tc-99m-Neu5Ac at 6.4 mM. These finding are important as C6 rat glioblastoma cells are highly resistant to cell death.[19] Reduction in viable cell count at higher concentration and long incubation time could be owed to the anti-inflammatory and antitumorigenic properties of sialic acid,[19] which generally comes into play at higher cellular uptake/concentration.

## Conclusion

The Neu5Ac was labeled with Tc-99m with maximum labeling efficiency of ≥90%. Cheminformatic studies conclude that there is negligible change in the ASA, PSA, and logP values of Tc-99m–Neu5Ac molecular complex. Number of hydrogen bond donors and acceptors are also in the permissible range according to the Pfizer rules and therefore, labeling Neu5Ac with Tc-99m does not disturb it's binding with the respective receptors *in vivo*. Further, the radiolabeled complex (Tc-99m-Neu5Ac) exhibits specific binding toward the C6 glioma cells with time- and concentration-dependent cytotoxicity. In conclusion, Tc-99m-Neu5Ac has the potential to be exploited as an *in vivo* radionuclide probe for tumor imaging.

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Nil.

### Conflicts of interest

There are no conflicts of interest.

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