CHAPTER 2

SYNTHESIS, RADIOLABELING, BIOLOGICAL EVALUATION OF COUMARIN COUPLED NITROIMIDAZOLES AS HYPOXIA MARKER

2.1 Cancer
The division of normal cells is controlled whereas cancerous cells replicate in uncontrolled way as shown in (fig. 2.1).

Figure 2.1: Uncontrolled cell division in cancer
Cancer is a collective term for numerous diseases, classified on cell type of origin, stage and where they reside. These different types of cancers distinguish themselves from normal cells by following characteristics:-

1. Unlimited proliferative capacity
2. Self-sufficient in growth factors
3. Escape apoptosis
4. Insensitive to anti-growth factors
5. Ability to invade surrounding tissue and metastasize
6. Acquire potential to induce angiogenesis
2.1.1 Genetic factor responsible for cancer

There are several genes (Ras oncogene and p53 tumor suppressor gene) involved in cellular transformation of normal cells to cancerous cells.

1. Tumor suppressor gene (p53)

These genes normally function to prevent abnormal cell growth/division by regulating cell cycle as shown in fig. 2.2 and thus involved in preventing cancer. It prevents the cell from completing cell cycle, if DNA is damaged. When this gene is mutated to cause a loss or reduction in its functionality, it results in cell progression towards cancer followed by combination of other genetic changes.

2. Oncogene(RAS)

These genes normally function to promote cell growth/division in an uncontrolled manner (Harris et al. 1991).

![Diagram](image)

**Figure 2.2:** Role of tumor suppressor gene and oncogene in cell cycle (Lehman et al. 1991)

2.1.2 Metastasis in cancer

Cancer cells inactivate p53 tumor suppressor gene (Vaupel et al. 1989). In normal cells reduction of telomeres (the protective ends of chromosomes), is responsible for controlled cell division whereas tumor cells expand with nonstop replicative potential by activating telomerase (Engelhardt and Martens 1998; Gordon and Parkinson 2004; Pan et al. 1997) as shown in fig. 2.3.
**Figure 2.3:** Telomerase and telomere activity in tumorigenesis (Fajkus et al. 2008)

Just like normal tissues, tumors also require nutrients and oxygen to grow (Dewhurst et al. 1994). Solid tumors activate angiogenesis (Kirsch et al. 2004), essential for organ growth, wound healing and tissue repair. But here it stands in contradiction to wound healing and tumor was described as “wound that is not healing”. It is also clear that human cell need 4 to 5 different mutations to become malignant (Kirsch et al. 2000) as depicted in fig. 2.4. Without adequate blood and nutrients, a tumour would not be able to grow, switch angiogenesis. However, Different combinations of mutations are acquired by different cancers, resulting in diverse behavior amongst similar types of cancer (Alberts et al. 2002).
**Figure 2.4:** Mutation, angiogenesis and metastasis in cancerous tissue (Folkman 2002)

Further, malignant tumor cells invade adjacent tissues and travel to distant sites to generate metastases, responsible for death due to most of cancers (Douglas and Robert 2000).

### 2.2 Hypoxia

As cancer is the main reason of death, it has resulted in numerous efforts by experts to localize, investigate and develop more individualized treatment to cure as well as to get better quality of life of affected patients. The available treatments included surgery, chemotherapy and radiotherapy, chosen depending on the tumor type, stage and condition of the patient. One of the parameter that directly influences efficacy of conventional cancer treatments is the presence of low oxygen regions called hypoxia. They observed viable tumor regions and as these regions were expanding, areas of necrosis appeared at the center represented in fig. 2.5(Jessica et al. 2008).

**Figure 2.5:** Necrotic regions in proliferated tumor

Based on these observations, they hypothesized that cells bordering the necrotic regions might be viable, but oxygen deprived or hypoxic as shown in fig. 2.6 (Brown and Wilson 2004).
Figure 2.6: Generation of hypoxia upon tumor expansion
Oxygen consumption in tumors increased many fold as a result of rapidly growing cancerous tissues, depends upon the efficiency of angiogenesis and neovascularization (Brahimi-Horn et al. 2007; Hockel and Vaupel 2001). As angiogenesis is vanished in cancer, it results in less oxygen and nutrient supply to the tumor (Boyle and Travers 2006). Thus, a proportion of cells in most tumors are exposed to relatively low oxygen tensions (pO₂ < 10 mmHg). While low oxygen potential can be lethal for cells, still significant numbers of tumor cells are still able to survive in poorly oxygenated (hypoxic) conditions. Hypoxia can be categorized into two main types: acute and chronic.

2.2.1 Acute (transient or perfusion-limited) hypoxia
It occurs due to abnormal blood vessels shutting down and then reopens, resulting in dramatic changes in perfusion. These temporal changes can occur several times within one hour, with intervals of 6 to 45 minutes.

2.2.2 Chronic (diffusion-limited) hypoxia
It arises when tumor outgrows their blood due to uncontrolled proliferation. As a result of this calculated distance at which oxygen diffuses is at average 100 μm (from 70 to 150 μm). However when cancer cells are located beyond this distance, they become necrotic. Chronically hypoxic cells are still viable and are located in the perinecrotic regions of the tumor (Pires et al. 2010).
The following characteristics, which are often perceived in tumors and contributing to impaired oxygen transport and hypoxia, are:-

1. Relatively sparse arteriolar supply
2. High O₂ consumption rate
3. Low vascular density
4. Inefficient orientation of blood vessels
5. Partial arteriolar supply
6. Sluggish blood flow due to stiffening of hypoxic RBC

Hypoxia regulates almost 80 genes associated with tumor angiogenesis and decrease immune reactivity (Ruan et al. 2009). Patients with high magnitude of hypoxic cells have less survival rate due to metastasis. Different degrees of hypoxia in different parts of organ and various cancer types are given in table 2.1.

**Table 2.1:** (a) Median values of pO₂ determined in different tissues

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Healthy Tissue</th>
<th>Median pO₂ (mm Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Breast</td>
<td>52</td>
</tr>
<tr>
<td>2.</td>
<td>Subcutis</td>
<td>51</td>
</tr>
<tr>
<td>3.</td>
<td>Skeletal muscle</td>
<td>37</td>
</tr>
<tr>
<td>4.</td>
<td>Cervix</td>
<td>42</td>
</tr>
<tr>
<td>5.</td>
<td>Pancreas</td>
<td>57</td>
</tr>
<tr>
<td>6.</td>
<td>Rectal mucosa</td>
<td>52</td>
</tr>
<tr>
<td>7.</td>
<td>Kidney</td>
<td>31</td>
</tr>
<tr>
<td>8.</td>
<td>Liver</td>
<td>30</td>
</tr>
<tr>
<td>9.</td>
<td>Brain</td>
<td>24</td>
</tr>
</tbody>
</table>

(c) Median values of pO₂ determined in diverse cancer types

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Neoplastic Tissue</th>
<th>Median pO₂ (mm Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Breast cancer</td>
<td>3 – 15</td>
</tr>
<tr>
<td>2.</td>
<td>K1735 malignant melanoma</td>
<td>37.8</td>
</tr>
<tr>
<td>3.</td>
<td>RENCA renal cell carcinoma</td>
<td>24.8</td>
</tr>
<tr>
<td>4.</td>
<td>Lewis lung carcinoma</td>
<td>1.8</td>
</tr>
<tr>
<td>5.</td>
<td>Cervix cancer</td>
<td>10</td>
</tr>
<tr>
<td>6.</td>
<td>Soft tissue carcinoma</td>
<td>20</td>
</tr>
<tr>
<td>8.</td>
<td>Squamous carcinoma</td>
<td>12.6</td>
</tr>
<tr>
<td>9.</td>
<td>Head &amp; neck carcinoma</td>
<td>4.8</td>
</tr>
</tbody>
</table>
2.3 Hypoxia Responsive gene

Hypoxia responsive genes are activated by hypoxia-inducible factor-1 [HIF-1] that binds to hypoxia-response elements (HREs), activating a genes to get better oxygen homeostasis shown in fig. 2.7 (Brahimini-Horn and Pouyssegur 2006, Calzado and del Paso 2007, Giatromanolaki and Harris 2001).

**Figure 2.7:** Role of HIF in normoxic and hypoxic condition (Qing and Simon 2009)
Figure 2.8: Consequences of hypoxia on various metabolic pathways

To summarize, hypoxic condition of tumors attributed to a poor prognosis due to treatment resistance (Gatenby et al. 1988; Wilson et al. 1992). Hypoxic tumor cells stay alive after chemotherapy and radiotherapy, so it is responsible for poor treatment outcomes. Radiotherapy fail due to low pO$_2$<5mmHg which reduce the accessibility of oxygen free radicals which are essential for DNA damage to lead cell death. The development of hypoxia marker increases the therapeutic percentage between tumor and normal tissue damage in patients (Brown 2007; Wilson and Hay 2011).

2.4 Detection of Hypoxia

A variety of approaches have been developed to measure tumor oxygenations (Chapman 1991; Moon et al. 2007) which are listed as: -

2.4.1 Polarographic oxygen electrode

A histogram show oxygen tensions (pO$_2$) distribution with low pO$_2$ measurements in tumor in fig. 2.9. (Hockel et al. 1996; Vaupel et al. 1991).
Figure 2.9: Polarographic measurements of tissue oxygen pressure in normoxic and hypoxic condition

2.4.2 $^{19}$F MRI

$^{19}$F NMR spectroscopy use perfluorocarbons (PFCs) to measure oxygen potential in biological systems (Mason et al. 2003). PFC allows injection of these compounds in large doses, as $^{19}$F NMR spin is highly sensitive to oxygen (Papadopoulou et al. 2006, Salmon and Siemann 2004). A number of PFCs have been surveyed and SR-4554 identified as the best with low toxicity (Aboagye et al. 1997, Koch et al. 1995, Papadopoulou et al. 2001).

2.5 Nitroimidazoles based hypoxia markers

Nitroaromatic compounds are used in cancer treatment as they increase the sensitivity of hypoxic regions to radiation/chemotherapy (Adams and Stratford 1986). Nitroaromatics are reduced by nitroreductase enzymes in hypoxic conditions as shown in fig. 2.10, it does not happen in necrotic tissue due to short of of enzyme activity (McClelland et al. 1987, Nunn et al. 1995, Overgaard 1994).

\[
\begin{align*}
\text{e}^- & \quad \text{RNO}_2 \quad \text{Nitroreductase} \quad \text{RNO}_2^- \\
\text{e}^- & \quad \text{RNO} \quad \text{RNOH} \quad \text{RNH}_2
\end{align*}
\]

Figure 2.10: Oxygen-dependent bioreductive metabolism of nitroimidazoles in hypoxic cells

The reaction is reversible in the presence of oxygen as shown in fig. 2.11 (Chapman et al. 1989; Kizaka-Kondoh and Konse-Nagasawa 2009).
**Figure 2.11:** Loss of nitroimidazole from well-oxygenated tissue

The biological properties of nitroaryl compounds are estimated by reduction potential. 2-nitroimidazole has higher reduction potential (-0.39 V) than 4-nitroimidazole (-0.56 V) and 5-nitroimidazole (-0.47 V) (Tocher and Edwards 1988).

![2-Nitroimidazole](image)

The rate of revival of parent compound depends upon oxygen because reduction potential of oxygen (-0.15 V) is higher than all nitroaryl compounds. If the oxygen concentration is low, intermediate like hydroxylamine, bind to proteins and DNA and nitro compounds are retained in the cells. Thus several 2-nitroimidazole derivatives have been identified as markers for imaging of tumor hypoxia (Chapman 1991). PET and SPECT techniques visualize hypoxic regions with the help of radiolabeled nitroimidazoles (Gilles et al. 2009). A number of nitroimidazoles compounds labeled with PET and SPECT radionuclides are described here:-

### 2.5.1 Positron Emission Computed Tomography (PET) Based Radiopharmaceutical

(a) **18**F-fluoromisonidazole (18FMISO):- (Graham et al. 1997; Koh et al. 1992; Prekeges et al. 1991; Rasey et al. 1996)

![18F-fluoromisonidazole](image)

(b) **18**F-Fluoroerythro-nitroimidazole (18F-FETNIM):- (Gronroos et al. 2001; Gronroos et al. 2004; Yang et al. 1995)
(c) $^{18}$F-EF5: (Barthel et al. 2004; Rasey et al. 1999; Zeimer et al. 2003)

(d) $^{18}$F-Fluoroazomycin arabinoside ($^{18}$F-FAZA): (Piert et al. 2005; Postema et al. 2009; Souvatzoglou et al. 2007)

(e) 2-$^{18}$F-Fluoro-2-Deoxy-D-glucose ($^{18}$F-FDG): (Clavo et al. 1995; Dierckx et al. 2008; Huang et al. 2012; Li et al. 2010; Pugachev et al. 2005)

(f) $^{64}$Cu(II)-diacetyl-2,3-bis(N4-methylthiosemicarbazone) ($^{64}$Cu-ATSM): (Bourgeois et al. 2011; Hansen et al. 2012; Obata et al. 2001; Yuan et al. 2006)

Thus numerous PET based markers are available in market for diagnosing hypoxia but the approach suffers due to:-
• It is an expensive technique.
• Use of radioactive markers.
• Positron Emitters usually have short half life of 2-100 minutes. This means that isotope must be available at the site of scanner using a cyclotron.
• It gives relatively poor spatial resolution and thus used along with CT or MRI.

2.5.2 Single Photon Emission Computed Tomography based Radiopharmaceutical (SPECT)

(a) Iodoazomycin arabinoside ($^{123}$I-IAZA):- (Mannan et al. 1991; Moore et al. 1993; Parliament et al. 1992)

![Iodoazomycin Arabinoside](image)

The uptake of $^{123}$I-IAZA is observed in patients with highly developed malignancies.

(b) $^{99m}$Tc labeled agents

1. BMS 181321

![BMS 181321](image)

2. BRU59-21

![BRU59-21](image)
3. \([^{99}\text{Tc}]\text{HL-91}\)

The *in vitro* and *in vivo* studies showed selective uptake of HL91 in hypoxic state as compared to controls (Iyer et al. 2001; Zhang et al. 1998).

Although SPECT is preferred over PET, as \(^{99}\text{Tc}\) has a number advantages:

- Low cost.
- Flexible half-life
- Flexible chemistry as compared to \(^{18}\text{F}\).
- Better spatial resolution

2.6 Dual Modality Imaging

Dual modality imaging is emerging as a method to improve the visual quality as well as increase the qualitative accuracy of radio imaging for diagnosis of a variety of diseases. For mechanistic studies of the uptake and bio-distribution in cells, the coupling of radio imaging with optical imaging is desirable. Biological chromophore result in cell auto-fluorescence predominantly in the blue region of the spectrum, and thus allow the visualization at the cellular level. The nitro group decrease the fluorescence of the ring system, but after bio-reduction in the hypoxic cells, the group fluoresces (Wardman et al. 1984). Nitroimidazoles with fluorescent groups on side chain were well reported as hypoxic cell markers (Hodgkiss et al. 1991). Naphthalimides (Middleton and Parrick 1985), Indolquinone (Komatsu et al. 2010) and Indolquinone-coumarins are potential fluorescent probes and localize preferentially in the hypoxic regions (Tanabe et al. 2008). For present invesigation coumarin moiety was selected as fluorescent label for imaging hypoxic cells because coumarins have diverse pharmacological, coordinating and fluorescence properties.
2.7 Coumarins as Fluorescent probe

Coumarin molecule (fig. 2.12) exhibits interesting fluorescence properties due to their intense fluorescence upon excitation above 330 nm, thus having application as fluorescent probes.

![Coumarin Molecular Structure](image)

**Figure 2.12:** Chemical structure of coumarin

Non-substituted coumarins exhibit very week fluorescence, however introduction of electron donating group (-OH, -OCH₃) at 7th position results in increase in fluorescence (Zhu et al. 2003). 7-Hydroxycoumarins have been on focus as fluorescent probes. 7-Hydroxycoumarins in organic solvents exhibit intense absorption band in the range of 320-390 nm and emission maximum between 390-460 nm respectively. The structures of commonly-used 7-Hydroxycoumarin as fluorescent marker are shown in Fig. 2.13.

![7-Hydroxycoumarin Derivatives](image)

7-Hydroxycoumarin-3-carboxylic acid 7-Hydroxycoumarin-3-carboxylic acid, succinimidyl ester

7-Hydroxycoumarin-3-acetic acid

**Figure 2.13:** Coumarin derivatives used as fluorescent probe

2.8 Coumarins as Complexing agent

Coumarins and their derivatives were found to exhibit good complexing ability with metal ions particularly lanthanides. A number of complexes of hydroxycoumarins with lanthanide ions have been studied spectrophotometrically and depending on pH 1:1, 1:2, 1:3 complexes are formed in solution (El-Ansary and Omar 1988; Ketata et al. 2012; Kostova et al. 2004; Mandakmre et al. 1997). The stability constants of the complexes of coumarin derivatives with La(III) increases with increasing atomic number.
2.9 Coumarins as Antitumor agent
Apart from acting as potential fluorophore, 7-hydroxycoumarin (HC) has been also regarded as an essential antitumor drug, due to their diverse pharmacological properties (Kostova et al. 2005). Coumarin is having low human toxicity and inhibits the growth of carcinoma (O’Kennedy and Lacy 2004; Jie et al. 2011; Oldenburg et al. 2008). The cytostatic effect of 7-hydroxycoumarin calculated in cancer cell lines like A549, SK-LU-1, 1.3.15, 3A5A. Thus coumarins are of great attention due to their physiological and anti-tumor activity. Numerous coumarin derivatives act as anticancer agents are given in fig. 2.14 (Kalluraya et al. 2000; Koneni et al. 2010; Musiliyu et al. 2009; Riveiro et al. 2008; Touisni et al. 2011).

![Coumarin derivatives](image)

**Figure 2.14**: Coumarin derivatives employed as anticancer agents
Coumarins inhibit cancer proliferation by inhibiting carbonic anhydrases, cyclin D and protein casein kinase (CK2α). Protein kinase is a potential target, where CK2α is known protein kinase, CK2 is involved in a numerous cell functions (Tawfic et al. 2001). Actually, CK2 are in abundance in tumors and cause alteration in the oncogenes or tumor suppressor genes (Zhu et al. 2010).

Polyphenolic compounds, like flavones, flavonols, anthraquinones and coumarins are probable kinase inhibitors as recommended by docking and the coumarins have been recognized as CK2 inhibitors (Adriana et al. 2008; Dan et al. 2010).

2.10 Molecular Docking
Linear interaction energy (LIE) approach used to estimate the binding energy of CK2 inhibitors (Huang et al. 2011; Zhang et al. 2010). Hydrophobic and hydrogen bonds are important for interactions between CK2 and its inhibitors, established by molecular docking. The results of molecular modeling forecast the activity of inhibitors which plays essential role in drug design.

2.11 Objectives
It was envisaged that 7-hydroxycoumarin coupled nitroimidazoles shall serve as hypoxia specific fluorescent imaging agent with therapeutic potential for inhibiting robust tumor growth. Two 7-hydroxycoumarin coupled nitroimidazole derivative 2NIHC and 4NIHC were synthesized.

Radiolabeling of both the compound and their biodistribution in EAT tumor bearing BALB/c mice have been done with 99mTcO₄⁻ to determine the T/M ratio. Flow cytometric analysis in A549 cell line has been done to calculate the normoxic-hypoxic differential of 2NIHC and 4NIHC. Inhibitory mechanism of coumarins toward CK2, glide software was used for docking of 7-Hydroxycoumarin analogues with protein kinase CK2 alpha. To evaluate the therapeutic
effectiveness of drugs tumor regression study on BALB/c mice as well as MTT assay have been carried out on A549 cell line.

2.12 Results and Discussion

2.12.1 Biological Characterization

(a) Flow cytometric Analysis

Before proceeding for their evaluation as a hypoxia marker, fluorescent spectra of 2NIHC and 4NIHC determined from a spectrofluorometer are illustrated in fig. 2.15, which indicates that the both fluorescent coumarins have emission wavelengths at 460 nm but the fluorescent intensity of 2NIHC is more than 4NIHC, which makes 2NIHC more suitable for flow cytometric studies.

![Fluorescence spectra](image)

**Figure 2.15:** Fluorescence spectra measured by spectrofluorometer for 2NIHC and 4NIHC

In vitro potency of 2NIHC and 4-NIHC was determined by flow cytometric analysis of cells incubated with both compounds under oxic and hypoxic conditions as shown in fig. 2.16.
**Figure 2.16:** Flow cytometric analysis of A549 cells incubated at 37°C with 10 mM of 2NIHC and 4NIHC for 4 h (a) control–hypoxic (b) control-normoxic* (c) 2NIHC-normoxic (d) 2NIHC-hypoxic (e) 4NIHC-normoxic (f) 4NIHC-hypoxic

*Hypoxic- (<1% O2)

Control- (21% O2)

There was approximately 10 fold increase in mean fluorescence in hypoxic condition when A549 cells were incubated with 2NIHC as compared to normoxic condition i.e from 864 to 8253. However, in the case of 4NIHC, only five time increase in uptake was noticed in hypoxic condition i.e 316 to 1423. The concentration dependent fluorescence in cells incubated with 2NIHC is shown in fig. 2.17. Maximum fluorescence intensity was observed with 10 mM concentration of 2NIHC, which indicates that drug binds firmly to the cellular macromolecules.

**Figure 2.17:** Mean fluorescence intensity from hypoxic and oxic cells incubated for 4 h at 37°C with 2NIHC

**(b) Cell Survival Activity of 2NIHC**

Cellular toxicity was determined by MTT assay carried out on A549 cells when treated with varying concentrations (10-0.0001μM) of 2NIHC at time intervals of 24, 48 and 72 h. With rising concentration of the compounds, the cell viability of A549 significantly decreased and data revealed 60% reduction in cell count when 0.1 μM of compound was incubated with A549 cells for 72 h. At lesser concentrations the compound did not show any undesirable effect on the cell survival as only 2% lyses of cell was observed when 0.0001 μM of the 2NIHC was incubated with A549 cell line for 24 h whereas 18% reduction in cell viability was noticed at 0.001 μM in 48 h. Afterward, 48% of cell lyses observed after 48 h exposure at 1 μM of the compound. After
48 h 2NIHC showed potent activity at 1 µM and % growth inhibition was 54%. The IC$_{50}$ value for the compound was found to be 1 µM as shown in figure 2.18.

![Graph showing cytotoxicity of 2NIHC](image)

**Figure 2.18:** Colorimetric estimation of the mitochondrial activity for cytotoxicity of 2NIHC (MTT assay) in A549 cell line (10-0.0001µM concentration range)

### 2.12.2 Computational Analysis

**(a) Linear Interaction Energy Model**

Molecular modeling is a key tool in drug designing to predict the binding modes of a ligand with protein. There are two aims of docking studies: accurate structural modeling and correct prediction of activity. Several important structural features like glide score, glide energy, interaction energy of H-bond, coulomb and vander walls energy score of coumarin derivatives are identified from their activity on CK2α. The ranking of ligands is based on glide score. As summarized in table 2.2, 2NIHC proved totally refractory to all compounds with higher glide score of -9.5 against protein kinase CK2α. It means, it can fit best in the receptor cavity and forming stable drug receptor complex. More the glide score, higher the inhibitory activity of ligand to proliferating tumor.

Primarily hydroxyl group at the 7 position of coumarin is considered as vital characteristic to increase the inhibitory potency. Analysis of the lipophilic contact term (-4.4) clearly reveals that the binding site is very hydrophilic and hydrophilic interactions dominate. The vander Waals is considered to be the main energy term favoring binding. Even with less favorable van der Waals energy for 10 (-40.1 kcal/mol) in comparison to 15 i.e. -50.6 kcal/mol, ligand 2NIHC achieves the highest glide score. The E$_{model}$ energy term value of -75.1 kcal/mol for the highest ranked ligand 10 shows that this is the dominant interaction controlling the ranking of a ligand. For
instance, in 4NIHC substitution of nitro group at position 4 results in loss of activity with glide score decrease from -9.5 to -3.6. Although the \( E_{vdW} \) and \( E_{coul} \) energy terms for 11 is higher than 10 but possess low glide score due to low \( E_{model} \) and \( H_{bond} \) values. However the contribution of \( H_{bond} \) for 10 is -2.1 which is highest among the other coumarin derivatives and revealed that there is good hydrogen bonding interaction between ligand 10 and the crystal structure of protein complex CK2\( \alpha \).

(b) Ligand-receptor docking

2D and 3D QSAR model of 2NIHC with CK2\( \alpha \) provide not only the relation between the molecular structure and their activity but also valuable clues for drug designing. As determined from the LIE model, the 2NIHC possesses the highest glide score, so it is docked with CK2\( \alpha \). A CK2\( \alpha \) inhibitor is small in size which makes it possible to lay out the residues in a 3D environment shown in fig. 2.19. A total of 19 residues in proximity of ligand along with hydrogen bonding interaction are shown in figure. The ligand is fitted into the active site by four hydrogen bonds, including acceptor contact with the sidechain of Glu114 and Val116 and a donor interface with backbone of Asp175 and Asn161 shown by purple arrows. Imidazole ring oriented parallel to protein structure for a possible \( \pi-\pi \) interaction with the His160.

Table 2.2: Molecular docking results of 2NIHC, 4NIHC compared with well known anticancer coumarin derivative against crystal structure of protein kinase CK2\( \alpha \) [PDB ID: 2Z JW]

<table>
<thead>
<tr>
<th>Compound</th>
<th>G* Score</th>
<th>EvdW</th>
<th>Ecoul</th>
<th>Emodel</th>
<th>Glide</th>
<th>Lipo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Hbond</td>
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</tr>
<tr>
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<td>-41.4</td>
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<td>-2.7</td>
</tr>
<tr>
<td>12(^a)</td>
<td>-7.3</td>
<td>-38.5</td>
<td>-5.6</td>
<td>-67.1</td>
<td>-1.0</td>
<td>-4.1</td>
</tr>
<tr>
<td>13(^b)</td>
<td>-6.6</td>
<td>-50.6</td>
<td>-8.9</td>
<td>-38.5</td>
<td>-1.3</td>
<td>-3.5</td>
</tr>
<tr>
<td>14(^c)</td>
<td>-5.5</td>
<td>-46.6</td>
<td>-3.0</td>
<td>-71.2</td>
<td>-0.5</td>
<td>-4.5</td>
</tr>
<tr>
<td>15(^d)</td>
<td>-7.1</td>
<td>-36.0</td>
<td>2.0</td>
<td>-51.5</td>
<td>0.0</td>
<td>-5.4</td>
</tr>
</tbody>
</table>
*Glide score is calculated using equation = 0.065*vdW + 0.130*Coul + Lipo + Hbond + Metal + Bury P + RotB + Site

Where Glide Hbond = Hydrogen-bonding term, Glide EvdW = Vander Waal energy, Glide Ecoul = Coulomb energy.

![2D Placement](image1) ![3D Active site](image2)

**Figure 2.19:** Cocrystal structure of CK2α (PDB code) with 10, rendered using default settings showing hydrogen bonds to side chains of Val 116 and Glu 114, to the backbone of Asp 175 and Asn 161, by purple arrows and interaction of imidazole group with His 160 by green.

### 2.12.3 Radiochemistry (Quality control of labeled conjugate)

The labeled complex remained at base and $^{99m}$TcO$_4^-$ moved along with solvent front. The labeling efficiency for $^{99m}$Tc-2NIHC and $^{99m}$Tc-4NIHC were calculated to be >99%, determined chromatographically. *In-vitro* stability of complexes, $^{99m}$Tc-2NIHC and $^{99m}$Tc-4NIHC were checked at time intervals of 4, 6, 24 and 48 h in PBS buffer at pH 7.0. The percentage labeling efficiency at 48 h was found to be 98%, implying that labeled conjugates were stable up to 48 h post labeling.

### 2.12.4 *In-vivo* study

**(a) Biodistribution study in mice bearing hypoxic tumor**

*In vivo* biodistribution studies of $^{99m}$Tc-2NIHC and $^{99m}$Tc-4NIHC were performed in BALB/c mice xenografted in their right leg with EAT tumors. The time course tissue uptake of $^{99m}$Tc-2NIHC at 1, 4 and 24 h was investigated and compared with $^{99m}$Tc-4NIHC at 4 h after tracer
injection, when maximum uptake takes place. Results obtained at 4 h post injection (p.i.) are summarized in Table 2.3 and 2.4. Clearance from background tissues at 1 h and 4 h through blood (4.89%ID/g and 1.39%ID/g) and muscle (0.32%ID/g and 0.23%ID/g) was seems to be more rapid, so that tumor/muscle ratio of $^{99m}$Tc-2NIHC increased with time and peak uptake value of 3.57(%ID/g) was observed at 4 h, while for $^{99m}$Tc-4NIHC it was 2.26(%ID/g) at 1 h p.i. The $^{99m}$Tc-2NIHC was delivered to the tumor more efficiently as compared to $^{99m}$Tc-4NIHC with significant washout over time (0.21%ID/g) after 24 h. In general, hydrophilic character of both radioconjugates increases their clearance via renal pathway, for the rapid clearance of background noise in images. Their kidney uptakes were high i.e. $22.81\pm1.02$ %ID/g at 4 h for $^{99m}$Tc-4NIHC and $37.99\pm1.09$ %ID/g at 4 h for $^{99m}$Tc-2NIHC indicative of extensive metabolism in the kidney. High uptake values of $^{99m}$Tc-4NIHC and $^{99m}$Tc-2NIHC in liver (19.46±1.12% and 13.27±1.04%) at 1 h indicates that they are eliminated from the body via renal as well as by hepatobiliary pathways. Uptake in the stomach was low (>1%) which shows good in vivo stability of both radioconjugates. It indicates that there is minimal, if any, in vivo dissociation of $^{99m}$Tc from these ligands to produce $^{99m}$TcO$_4^-$.

**Table 2.3:** Biodistribution of $^{99m}$Tc-4NIHC in BALB/c mice bearing EAT hypoxic tumor at 4 h p.i.$^a$

<table>
<thead>
<tr>
<th>Organ</th>
<th>%ID/g</th>
<th>1 hr</th>
<th>4 h</th>
<th>24 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>5.31±0.02</td>
<td>2.34±0.04</td>
<td>0.73±0.03</td>
<td></td>
</tr>
<tr>
<td>Heart</td>
<td>1.23±0.01</td>
<td>0.76±0.01</td>
<td>0.23±0.03</td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>17.39±1.08</td>
<td>22.81±1.02</td>
<td>12.41±0.86</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>19.46±1.12</td>
<td>16.16±0.98</td>
<td>9.71±0.88</td>
<td></td>
</tr>
<tr>
<td>Lungs</td>
<td>3.54±0.11</td>
<td>2.03±0.08</td>
<td>0.93±0.05</td>
<td></td>
</tr>
<tr>
<td>Stomach</td>
<td>0.91±0.12</td>
<td>0.41±0.04</td>
<td>0.12±0.01</td>
<td></td>
</tr>
<tr>
<td>Muscle</td>
<td>0.85±0.02</td>
<td>0.41±0.01</td>
<td>0.14±0.01</td>
<td></td>
</tr>
<tr>
<td>Tumor</td>
<td>1.72±0.02</td>
<td>0.93±0.01</td>
<td>0.21±0.01</td>
<td></td>
</tr>
<tr>
<td>T/M ratio</td>
<td>2.02±0.02</td>
<td>2.26±0.01</td>
<td>1.5±0.01</td>
<td></td>
</tr>
</tbody>
</table>

$^a$Values are expressed as the mean ± SD; n = 3.
Table 2.4: Biodistribution of $^{99m}$Tc-2NIHC in BALB/c mice bearing EAT hypoxic tumor at 4 h p.i.$^{a}$

<table>
<thead>
<tr>
<th>Organ</th>
<th>1hr</th>
<th>4 h</th>
<th>24hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>4.89±0.12</td>
<td>1.39±0.14</td>
<td>0.24±0.07</td>
</tr>
<tr>
<td>Heart</td>
<td>0.55±0.2</td>
<td>1.35±0.11</td>
<td>0.13±0.08</td>
</tr>
<tr>
<td>kidney</td>
<td>29.60±1.04</td>
<td>37.99±1.09</td>
<td>17.73±0.95</td>
</tr>
<tr>
<td>Liver</td>
<td>13.27±1.04</td>
<td>9.05±0.68</td>
<td>1.47±0.22</td>
</tr>
<tr>
<td>Lungs</td>
<td>0.73±0.14</td>
<td>5.69±0.71</td>
<td>2.46±0.08</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.85±0.14</td>
<td>0.52±0.12</td>
<td>0.18±0.08</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.32±0.02</td>
<td>0.23±0.01</td>
<td>0.07±0.02</td>
</tr>
<tr>
<td>Tumor</td>
<td>0.59±0.02</td>
<td>0.83±0.01</td>
<td>0.20±0.02</td>
</tr>
<tr>
<td>T/M ratio</td>
<td>1.82±0.02</td>
<td>3.57±0.01</td>
<td>2.97±0.02</td>
</tr>
</tbody>
</table>

$^{a}$Values are expressed as the mean ± SD; n = 3.

(b) Scintigraphy in mice bearing normal and hypoxic tumor

The selection of only $^{99m}$Tc-2NIHC for scintigraphic imaging was based on higher tumor uptake as depicted from biodistribution data. The 4 h p.i. whole body image of the $^{99m}$Tc-2NIHC showed uptake in the hypoxic tumor sites as well as in kidneys, liver, intestine and bladder in decreasing intensity relative to the background tissues. Hypoxic tumor grafted in BALB/c mice clearly identifiable in the γ camera images (fig. 2.20a) and shows high target to non target ratio of $^{99m}$Tc-2NIHC. Hypoxic tumor site was also blocked with a large excess of 2NIHC in one set of mice to evaluate its uptake at 4 h p.i. As shown in fig. 2.20b, the image of the mice with blocked hypoxic tumor site gave no significant uptake in the tumor sites. Similarly the uptake of radiotracer in mice bearing normal tumor is also negligible as shown in fig. 2.20c, thus $^{99m}$Tc-2NIHC has high affinity for hypoxic tumor site.
Figure 2.20: Whole-body γ image of BALB/c mice with tumor in right thigh (red circle indicate tumor site) at 4 h p.i. (a) In mice bearing hypoxic tumor without blocking (b) In mice bearing hypoxic tumor with blocking (c) In normal tumor bearing mice. Planar images were acquired in anterior position

2.12.5 Antitumor Screening

Animal studies were carried out using an EAT bearing BALB/c mice model. The growth of tumor was observed for 30 days after treatment with 2NIHC at a dose of 30 mg/kg body weight and also without 2NIHC which was considered as a control group. 2NIHC controlled tumor growth significantly and thus constitutes an interesting candidate for anticancer drug. Coumarin compounds possess anticancer activity, which makes 2NIHC suitable for assessment of its anticancer activity. The inhibitor concentrations were tolerated well with no significant reduction in weight of any of the mice during the tumor regression study. As shown in fig. 2.21 the average tumor volume on day 31st was 1550 mm³ when treated with 1mg/ml of 2NIHC and 2500 mm³ in the case of control group. The result indicates that 2NIHC reduced the tumor growth as compared to control. Thus, 2NIHC inhibited the growth of tumors at dose of 30 mg/kg and increased the survival time.

Figure 2.21: Tumor growth inhibition in EAT-bearing BALB/c mice and data represent mean of five animals. 2NIHC was given at a dose of 30 mg/kg on 15th, 17th, 19th and on 23rd day. Control was considered without any vehicle
2.13 Conclusion
We have successfully synthesized and evaluated $^{99m}$Tc-2NIHC and $^{99m}$Tc-4NIHC for the visualization of hypoxic tumor by radiometric analysis and fluorescence microscopy. The molecule also possesses valuable therapeutic potential. Strong intrinsic fluorescence intensity of 2NIHC coupled with solubility and stability in biologically compatible medium facilitated the monitoring of delivery to cells. The *in vivo* biodistribution and imaging studies $^{99m}$Tc-2NIHC in hypoxic tumor showed high uptake with quick elimination of radioactivity from the bloodstream via the liver and kidney. Tumor regression study, MTT assay and molecular docking studies explore the high inhibitory mechanism of 2NIHC than 4NIHC for hypoxic tumor.
2.14 Experimental

2.14.1 Synthesis

Figure 2.22: Synthesis route of 2-(7-Hydroxy-2-oxo-2H-chromen-4-yl)-N-{2-[4-(2/4-nitroimidazole-1-yl)-butylamino]-ethyl}-acetamide
Reagents: - (a) (boc)₂O, TEA, 0°C–rt, 88%; (b) 7-hydroxycoumarinyl-4-acetic acid, HOBT, DCC, 90%; (c) TFA, 0°C, 72%; (d) 1,4-dibromobutane, K₂CO₃, 70°C, 75-80%; (e) TEA, 70°C, N-(2-Amino-ethyl)-2-(7-hydroxy-2-oxo-2H-chromen-4-yl)-acetamide, 60-70%.

Synthesis of (2-Amino-ethyl)-carbamic acid tert-butyl ester (2)

To a stirred solution of ethylenediamine 1 (3.06 ml, 45.85 mmol) in ethanol at 0°C triethylamine (1.28 ml, 9.17 mmol) was added. The solution of boc-anhydride (2.00 g, 9.17 mmol) dissolved in 10 ml of ethanol was added dropwise to the reaction mixture. The reaction mixture stirred for 24 h and then solvent was evaporated. The reaction mixture was extracted with chloroform/water and organic layers were dried over anhydrous sodium sulphate, evaporated to obtain the transparent oily product (5.32 g, 79%). ¹H NMR (400MHz, CDCl₃) δH:- 1.38 (s, 9H, CH₃), 1.54 (brs, NH₂), 2.73 (t, 2H, CH₂), 3.10 (t, 2H, CH₂), 5.19 (brs, NH). ¹³C NMR (100MHZ, CDCl₃) δC: - 28.35 (CH₃), 41.79, 43.32 (CH₂), 79.07, 156.27 (C=O). MS (ESI): m/z calculated 160.1, found 161.2[M+H]+, 105.5 [M– (C(CH₃)₃)]+.

Synthesis of {2-[2-(7-Hydroxy-2-oxo-2H-chromen-4-yl)-acetylamino]-ethyl}-carbamic acid tert-butyl ester (4)

A solution of 7-hydroxy coumarinyl-4-acetic acid 3 (0.99 g, 4.49 mmol) and HOBT (0.60 g, 4.49 mmol) in dry DMF (25 ml) was stirred at 0°C and a solution of DCC (1.01 g, 4.93 mmol) in dry DMF (5 ml) was added. Compound 2 (0.72 g, 4.49 mmol) was added at 0°C to the reaction mixture and stirred for 2 h and then reaction mixture was stirred at rt for 24 h. The DCU (dicyclohexylurea) was filtered off and solvent was concentrated. The concentrate was dissolved in 1 ml of MeOH followed by addition of chloroform dropwise. The compound get precipitated out as pale white solid and purified by silica gel chromatography (20% methanol in chloroform with a yield of 1.35 g, 70%. ¹H NMR (400MHz, DMSO) δH:- 1.37 (s, 9H, CH₃), 2.98 (t, 2H, CH₂), 3.08(t, 2H, CH₂), 3.62 (s, 2H, CH₂), 6.16 (s, 1H, CH), 6.71 (d, 1H, Jpara=2.4 Hz, CH), 6.79 (dd , 1H, J₁=8.4 Hz, J₂=8.8 Hz, CH), 7.59 (d, 1H, J=8.4 Hz, CH). ¹³C NMR (100MHz, DMSO) δC:- 28.67, 39.22-40.55 (3×CH₂), 78.18, 102.71, 111.95, 112.28, 113.38, 127.20, 151.56, 155.45, 156.10, 160.73, 161.62 (C=O), 168.30 (C=O). MS (ESI): m/z calculated 363.1, found 362.1 [M-H]⁻.

Synthesis of N-(2-Amino-ethyl)-2-(7-hydroxy-2-oxo-2H-chromen-4-yl)-acetamide (5)
The tert-butyl group of compound 4 (1.00 g, 2.75 mmol) was deprotected by trifluoroacetic acid (5 ml) in anhydrous dichloromethane at 0°C and followed by stirring at room temperature for 6 hr. The solvent was evaporated to obtain the crude product. The product obtained washed with methanol (3×20 ml). Addition of diethyl ether precipitate product as white solid (0.66 g, 69% yield). \( ^1H \) NMR (400MHz, D\(_2\)O) \( \delta \): 3.01 (t, 2H, CH\(_2\)), 3.40 (t, 2H, CH\(_2\)), 3.72 (s, 2H, CH\(_2\)), 6.11 (s, 1H), 6.64 (s, 1H, CH), 6.74 (d, 1H, J=8.0 Hz, CH), 7.40 (d, 1H, J=8.0 Hz, CH). \( ^{13}C \) NMR (100MHz, D\(_2\)O) \( \delta \_c \): 37.04, 38.47, 38.88, 102.80, 112.02, 112.05, 113.58, 126.22, 151.34, 154.51, 160.07, 164.03 (C=O), 172.04 (C=O). MS (ESI): calculated 262.1, found 261.1 [M-H]; 175.1 [M-CH\(_2\)CONH(CH\(_2\)_2)NH\(_2\)]

**Synthesis of 1-(4-Bromo-butyl)-4(or 2)-nitro-1H-imidazole (8, 9)**

A solution of 4-nitroimidazole and 2-nitroimidazole (1.00 g, 8.92 mmol) and K\(_2\)CO\(_3\) (9.84 g, 71.36 mmol) in acetonitrile (50 ml) was stirred at 70°C for 0.5 hr followed by the addition of 1, 4-dibromobutane (4.19 ml, 35.68 mmol). After completion, reaction mixture was filtered, evaporated and purified by silica gel chromatography (10% methanol in chloroform). The product was crystallized as pale yellow crystal of 4-nitroimidazole derivative (1.22 g, 76% yield) and yellow powder of 2-nitroimidazole derivative (1.31 g, 79% yield) in hexane.

(8) 2-nitroimidazole derivative

\( ^1H \) NMR (400MHz, D\(_2\)O) \( \delta \): 1.92 (q, 2H), 2.14 (q, 2H), 3.41 (t, 2H, CH\(_2\)), 4.46 (t, 2H, CH\(_2\)), 7.13 (d, 2H, J=6.0 Hz). \( ^{13}C \) NMR (100MHz, D\(_2\)O, Me\(_4\)Si): \( \delta \_c \) = 29.29, 30.93, 32.38, 49.43, 126.02, 128.52. MS (ESI): m/z calculated 247.0, found 248 and 250 [M+H]\(^+\) with isotopic pattern of Br.

(9) 4-nitroimidazole derivative

\( ^1H \) NMR (400MHz, D\(_2\)O) \( \delta \): 1.92 (q, 2H), 2.04 (q, 2H), 3.42 (t, 2H, CH\(_2\)), 4.12 (t, 2H, CH\(_2\)), 7.48 (s, 1H), 7.83 (s, 1H). \( ^{13}C \) NMR (100MHz, D\(_2\)O) \( \delta \_c \) ppm: 29.15, 29.26, 32.29, 47.60, 119.35, 136.06, 148.11. MS (ESI): calculated 247.0, found 248.0 and 250.0 [M+H]\(^+\), 270.0 and 272.2 [M+Na]\(^+\) with isotopic pattern of Br.

2-(7-Hydroxy-2-oxo-2H-chromen-4-yl)-N-{2-[4-(2 or 4-nitroimidazole-1-yl)-pentyloamino]-ethyl}-acetamide (10, 11)
Triethylamine (0.15 ml, 1.14 mmol) was added to the stirred solution of 5 (0.20 g, 0.76 mmol) in water. It was stirred for 15 min followed by the addition of 8 (0.18 g, 0.72 mmol) and heated to 80°C. Reaction was continued for 24 hr and the progress of the reaction checked by TLC. Reaction mixture was evaporated, dissolved in small amount of methanol and get precipitated as light brown solid with diethyl ether. Yield of 4-nitroimidazole derivative and of 2-nitroimidazole derivative was (0.19 g, 60% yield), (0.24 g, 68% yield) respectively.

(10) 2-nitroimidazole derivative

$^1$H NMR (400MHz, D$_2$O) δ$_H$: 1.59-1.79 (m, 4H, CH$_2$), 2.95-3.44 (m, 6H, CH$_2$), 3.63 (s, 2H, CH$_2$), 4.30 (t, 2H, CH$_2$), 6.01 (s, 1H, CH), 6.35 (brs, 1H, CH), 6.60 (brs, 1H, CH), 7.00 (brs, 1H, CH), 7.29 (brs, 2H, CH). $^{13}$C NMR (100MHz, D$_2$O) δ$_C$: 26.22, 28.21, 46.99, 49.30, 52.69, 60.99, 102.66, 111.81, 113.68, 126.30, 127.71, 127.90, 144.18, 151.31, 154.18, 160.35, 161.66, 163.69, 171.70. MS (ESI): m/z calculated 429.1 and found 430.2 [M]$^+$.  

(11) 4-nitroimidazole derivative

$^1$H NMR (400MHz, DMSO) δ$_H$: 1.69 (q, 2H, CH$_2$), 1.94 (q, 2H, CH$_2$), 2.39 (s, 2H, CH$_2$), 2.50 (t, 4H, CH$_2$), 4.09 (t, 2H, CH$_2$), 6.01 (s, 1H, CH), 6.71 (br, s, NH), 6.94 (s, 1H, CH), 6.96 (d, 1H, J=10.8 Hz, CH), 7.35 (br, s, 1H, NH), 7.67 (d, 1H, J=8.8 Hz, CH), 7.90 (d, 1H, J=1.2 Hz, CH), 8.46 (d, 1H, J=1.2 Hz, CH). $^{13}$C NMR (100MHz, DMSO) δ$_C$: 25.68, 27.13, 47.50, 68.02, 101.62, 111.53, 112.95, 113.55, 122.03, 126.94, 137.90, 147.41, 154.01, 155.16, 162.02, 172.24. MS (ESI): m/z calculated 430.1 and found 431.2[M+H]$^+$, 495.8[M+Na+K]$^+$.  

2.14.2 In-vitro Study

(a) Cell uptake by Flow Cytometry

To evaluate cell uptake, exposure of hypoxia was given to A549 cell line (<1% O$_2$) in a hypoxic chamber. Hypoxic and normoxic A549 cells were incubated with 2NIHC and 4NIHC for 4h at different concentration of 10 mM, 10 μM and 10 nm. After incubation cells washed with PBS twice, harvested using 0.5 ml of trypsin for 5 minutes and resuspended in PBS. 6 μl of 1 μg/ml
of propidium iodide in PBS was added, incubated for half an hour in the dark at -4 °C and then samples analyzed by Flow cytometer FACS calibur.

(b) Cytotoxicity of 2NIHC

Mitochondrial activity was expressed as percentage of surviving cells

% of viability = [OD test product/OD negative control] × 100%.

Percentage viability plotted against varying concentrations (10-0.0001µM) of compound (2NIHC) at different time interval.

2.14.3 Computational Analysis

Over actvation of receptor protein kinase CK2α is a key factor in tumor genesis. Thus there is increasing interest on the deactivation of CK2α to inhibit the growth of cancerous cells. The human CK2 catalytic subunit was built using a homology modeling approach with α isoform as template (PDB code ID: 2ZJW). In order to determine binding mode of the 2NIBC with CK2α it is docked with structure of CK2α.

(a) Linear Interaction Energy Model

Human protein kinase CK2α complexed with [PDB ID: 2ZJW] was taken for molecular docking studies and best pose chosen from Glide score as well as from interactions between the ligands and active site of amino acids.

(b) Ligand-Receptor Docking

Docking experiments were carried out by using GLIDE (grid based ligand docking with energetic) and ligand docking programme. The docking was initiated with putting specified receptor grid and prepared ligand molecule together.

2.14.4 Radiochemistry

(a) ⁹⁹ᵐTc Radiocomplexation of 2NIHC and 4NIHC

The ligand i.e. 2NIHC and 4NIHC (4.6 mmol) was dissolved in 1ml of double distilled water. 100 µl of this solution was taken in a vial and stannous chloride (1 µmol) was added to it
followed by the addition of freshly eluted $^{99m}$-Technecium pertechnetate (2 mCi; 200μl) saline solution and vial was sealed. The pH was adjusted to 7 using 0.1 M sodium carbonate solution. All contents were thoroughly mixed and vial was kept at rt for 0.5 h.

(b) Radiochemical purity of $^{99m}$Tc-2NIHC and 4NIHC complex

The radiolabeling efficiency of above complexes was resolute by using instant thin layer chromatography using 100% acetone or pyridine/acetic acid/water (PAW; 3:5:1.5). TLC strips were cut into fragments and counts of each fragment were taken. The complexed $^{99m}$Tc remained at the origin whereas free $^{99m}$Tc moved with the solvent.

(c) Serum stability of complex

To determine the stability of the labeled complex, human serum was incubated with $^{99m}$Tc radiolabeled complex at 37°C for 1 h in a incubator maintained at 5% CO$_2$ and 95% air. The radiolabeled conjugate was then analyzed for any dissociation x by ITLC strips with 100% acetone and pyridine, acetic acid and water (PAW) (3:5:1.5) as a mobile phase. Free pertechnetate at different time intervals was estimated, which gives the percentage labeling efficiency or dissociation of the complex.

2.14.5 In-vivo Study

All animal experiments were repeated in triplicates and done in accordance with the guidelines of animal ethics committee. EAT tumor bearing BALB/c mice were used for tumor regression study, biodistribution, blood clearance and gamma imaging.

(a) Biodistribution Study in mice bearing hypoxic tumor

This study was done after 12 days of inoculation of EAT cells into BALB/c mice, so that hypoxic conditions were generated in tumor for its assessment. Approximately 2 mCi of $^{99m}$Tc-2NIHC and $^{99m}$Tc-4NIHC was injected intravenously into tumor bearing BALB/c mice through tail. At 1, 4 and 24 h post injection, mice were sacrificed and biodistribution carried out in tumor and in lung, liver, kidney, stomach, blood and muscle. The selected tissues and organs were harvested and weighed. Uptake of $^{99m}$Tc radioactivity in each sample was measured as %ID/g of the tissue with an automated gamma scintillation counter and was normalized with $^{99m}$Tc-decay correction.
(b) Scintigraphy in mice bearing normal and hypoxic tumor

As it was cleared from the biodistribution studies that 2NIHC accumulates more in the hypoxic tumor than 4NIHC along with fast clearance from the body. Therefore scintigraphic imaging of mice bearing hypoxic tumor was done with only 2NIHC. After intravenous injection of 2 mCi of $^{99m}$Tc-2NIHC in mice bearing hypoxic tumor, animals were subjected to SPECT imaging at time intervals of 1, 4 and 24 h post injection. The effect of $^{99m}$Tc-2NIHC and $^{99m}$Tc-4NIHC was also observed in normal tumor bearing mice where tumor was ~1 week old. Image acquired at 4 h post injection delivered the maximum drug uptake in hypoxic tumor site and at same time uptake was also noticed for normal tumor bearing mice.

(c) Antitumor Screening

7-Hydroxy coumarin and their derivatives are inhibitor of tumor, hence to evaluate l inhibition of tumor growth in vivo, mice bearing EAT tumor were treated with 2NIHC. All animals were in a pathogen-free environment. As molecular docking studies have shown that 2NIHC is more potent anticancerous drug than 4NIHC. Mice were divided into two groups (control, 2NIHC) with each group having five. After administration of cancer cells, tumors were allowed to grow for 3 weeks till they become hypoxic and then treatment was initiated. In one set of group (n=5), 4NIHC (1 mg/ml) was administered on the 15th day of implantation of tumor by intravenous injection via a lateral tail vein at a dose of 30mg/kg and another set was taken as control, which was without any vehicle. The dosing plan implicated with injections every alternate day for doses 2 and 3 i.e. on 17th and 19th, followed by injection every third day i.e. 23rd and 27th day for remaining doses. Tumor diameters were measured on days 17, 19, 23, 27 and 31 with vernier calipers and tumor volume was measured using formula (LxW$^2$/2). The study was conducted up to 30 days.
Characterization Data of 2NIHC and 4NIHC
$^1$H NMR spectra of (2-Amino-ethyl)-carbamic acid tert-butyl ester (2)

$^{13}$C NMR spectra of (2-Amino-ethyl)-carbamic acid tert-butyl ester (2)

Mass spectra of (2-Amino-ethyl)-carbamic acid tert-butyl ester (2)
$^1$H NMR spectra of 2-[2-(7-Hydroxy-2-oxo-2H-chromen-4-yl)-acetylamino]-ethyl]-carbamic acid tert-butyl ester (4)
$^{13}$C NMR spectra of [2-[2-(7-Hydroxy-2-oxo-2H-chromen-4-yl)-acetylamino]-ethyl]-carbamic acid tert-butyl ester (4)

Mass spectra of [2-[2-(7-Hydroxy-2-oxo-2H-chromen-4-yl)-acetylamino]-ethyl]-carbamic acid tert-butyl ester (4)
$^1$H NMR spectra of N-(2-Amino-ethyl)-2-(7-hydroxy-2-oxo-2H-chromen-4-yl)-acetamide (5)

$^{13}$C NMR spectra of N-(2-Amino-ethyl)-2-(7-hydroxy-2-oxo-2H-chromen-4-yl)-acetamide (5)
Mass spectra of N-(2-Amino-ethyl)-2-(7-hydroxy-2-oxo-2H-chromen-4-yl)-acetamide (5)

Fragment spectra of N-(2-Amino-ethyl)-2-(7-hydroxy-2-oxo-2H-chromen-4-yl)-acetamide at 175 (5)
$^1$H NMR spectra of 1-(4-Bromo-butyl)-2-nitro-1H-imidazole (8)

$^{13}$C NMR spectra of 1-(4-Bromo-butyl)-2-nitro-1H-imidazole (8)
Mass spectra of 1-(4-Bromo-butyl)-2-nitro-1H-imidazole (8)

\[
[M+H]^+ 
\]

\[\text{Mass spectrum of 1-(4-Bromo-butyl)-2-nitro-1H-imidazole (8)}\]

\[\text{N.M. O. 0.1-0.2min. R(3-5)}\]

\[\text{N.H. O. 0.1-0.2min. R(3-5)}\]

\[\text{1H NMR spectra of 1-(4-Bromo-butyl)-4-nitro-1H-imidazole (9)}\]

\[\text{1H NMR spectra of 1-(4-Bromo-butyl)-4-nitro-1H-imidazole (9)}\]
$^{13}$C NMR spectra of 1-(4-Bromo-butyl)-4-nitro-1H-imidazole (9)

Mass spectra of 1-(4-Bromo-butyl)-4-nitro-1H-imidazole (9)
$^1$H-NMR spectra of 2-(7-Hydroxy-2-oxo-2H-chromen-4-yl)-N-[2-[4-(2-nitroimidazole-1-yl)-pentylamino]-ethyl]-acetamide (10)

$^{13}$C NMR spectra of 2-(7-Hydroxy-2-oxo-2H-chromen-4-yl)-N-[2-[4-(2-nitroimidazole-1-yl)-pentylamino]-ethyl]-acetamide (10)
Mass spectra of 2-(7-Hydroxy-2-oxo-2H-chromen-4-yl)-N-{2-[4-(2-nitroimidazole-1-yl)-pentylamino]-ethyl}-acetamide (10)

\[ \text{Mass spectra of 2-(7-Hydroxy-2-oxo-2H-chromen-4-yl)-N-{2-[4-(2-nitroimidazole-1-yl)-pentylamino]-ethyl}-acetamide (10)} \]

\[ \text{\textsuperscript{1}H NMR spectra of 2-(7-Hydroxy-2-oxo-2H-chromen-4-yl)-N-{2-[4-(4-nitroimidazole-1-yl)-pentylamino]-ethyl}-acetamide (11)} \]

\[ \text{\textsuperscript{1}H NMR spectra of 2-(7-Hydroxy-2-oxo-2H-chromen-4-yl)-N-{2-[4-(4-nitroimidazole-1-yl)-pentylamino]-ethyl}-acetamide (11)} \]
\textsuperscript{13}C NMR spectra of 2-(7-Hydroxy-2-oxo-2H-chromen-4-yl)-N-{2-[4-(4-nitroimidazole-1-yl)-pentylamino]-ethyl}-acetamide (11)

[Graph of NMR spectrum]

Mass spectra of 2-(7-Hydroxy-2-oxo-2H-chromen-4-yl)-N-{2-[4-(4-nitroimidazole-1-yl)-pentylamino]-ethyl}-acetamide in negative mode (11)

[Graph of mass spectrum]
Mass spectra of 2-(7-Hydroxy-2-oxo-2H-chromen-4-yl)-N-{2-[4-(4-nitroimidazole-1-yl)-pentylamino]-ethyl}-acetamide in positive mode (11)
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