CHAPTER V

Antitumor Activity of N-Arylsubstituted Hydroxamic Acids
The antitumor activity of nineteen N-arylsubstituted hydroxamic acids is reported in this chapter. The antiproliferative effect of hydroxamic acids against human breast cancer (MCF-7) cells were determined using MTT assay in microliter plates. IC₅₀ values obtained were further used for QSAR study.
ANTITUMOR ACTIVITY OF N-ARYLSUBSTITUTED HYDROXAMIC ACIDS

CANCER BIOLOGY

Cancer cells in culture condition
CANCER BIOLOGY

Cancer is characterized by unlimited growth and proliferation of cells. Cancer is the second leading cause of death in the world. Cancer is a genetic disorder involving dynamic changes in the genome leading to uncontrolled cell growth, ability to invade and metastasize. The genes implicated in cancer include those involved in cell cycle control, apoptosis, DNA repair, aging and immortalization and metastasis.

Normally the division and growth of cells are orderly and controlled, but if this process loses its control, the cells will continue to divide into lump, which is called a tumor. Tumors can either be benign or malignant. Cancer is the name given to a malignant tumor.

In a benign tumor the cells do not spread to other parts of the body and so are cancerous. However, if they continue to grow and at the original site, they may cause problems by pressing on the surrounding organs. A cancerous tumor consists of cancer cell, which have the ability to spread beyond the original site. Malignant tumor can break off main tumor and travel through the blood stream or lymphatic system until they find a suitable place to start forming a new tumor. This process is called metastasis.

Cancer cells have two heritable properties,

- They and their progeny reproduce in defiance of the normal restraints.
- Invade and colonize territories normally required for other cells. These features make cancer peculiarly dangerous.
Cancers are classified by the type of cell that resembles the tumor and therefore, the tissue presumed to be the origin of the tumor. The following general categories are usually accepted:

- **Carcinoma**: malignant tumors derived from epithelial cells. This group represents the most common cancers, including the common forms of breast, prostate, lung and colon cancer.
- **Lymphoma and Leukemia**: malignant tumors derived from blood and bone marrow cells.
- **Sarcoma**: malignant tumors derived from connective tissue or mesenchymal cells.
- **Mesothelioma**: tumors derived from the mesothelial cells lining the peritoneum and the pleura.
- **Glioma**: tumors derived from glia, the most common type of brain cell.
- **Germinoma**: tumors derived from germ cells, normally found in the testicle and ovary.
- **Choriocarcinoma**: malignant tumors derived from the placenta.

**Breast cancer** is the most frequent cancer and the second leading cause of cancer death in woman today (156,157). According to World Health Organization, more than 1.2 million people are diagnosed with breast cancer this year worldwide, even though the incidence leveled off and the death rate of breast cancer declined significantly after the 1990s. Medical experts attribute the decline in breast cancer deaths to earlier detection and more effective treatments (158,159). Estrogens are well recognized to play the predominant role in breast cancer development and growth, and most of the
efforts have been devoted to block estrogen formation and action (157,160). The most widely used therapy for breast cancer is the use of an antiestrogen such as tamoxifen (TAM). However, the present breast cancer therapies achieve meaningful clinical results in only 30-40% of patient (157), because drug resistance usually develops after one or two years of treatment. The resistance is linked to the presence of estrogen-independent pathways for breast cancer cells growth (161,162). Recently, it was reported that expression of the human estrogen receptor -2(HER-2) is also significant factor associated with breast cancer morbidity (163). It is also becoming clearer that cross-talk between estrogen and growth factor receptor pathway occurs and likely is a factor in the pathology and treatment of breast (164). Therefore, more potent anti-breast cancer agents combine the desired, tissue selective effects with novel structure or new mechanism of action must be developed. With this view in mind, the present investigation is undertaken.

EXPERIMENTAL SECTION

MATERIALS

Dulbecco’s Modified Eagle’s Medium (DMEM) was purchased from GibCO, NY, USA and fetal bovine serum (FBS) was obtained from Biomedia, France. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] was obtained from Sigma, USA. Dimethy sulphoxide was purchased from Merck (Darmstadt, Germany).
CELL SYSTEM

MCF-7: MCF-7 are human epithelial cells from human breast adenocarcinoma established from the pleural effusion of a 69 year old caucasian woman with metastatic mammary carcinoma (after radio- and hormones therapy) in 1970. MCF-7 is adherent cells which maintain contact inhibition in-vitro.

Cell line: MCF-7
Origin: Epithelial like cells growing as monolayer
MCF-7: 90% DMEM supplemented with 10% FCS, penicillin (100 units/ml) and streptomycin (250μg/ml).
Sub cultivation ratio: A sub cultivation ratio 1:3 to 1:6 is recommended.
Doubling time: 29 hrs.
Preservation: Frozen with 70% medium dimethyl sulphoxide (DMSO) at about 3x10^6 cells /cryovial
Storage temperature: Liquid nitrogen vapour phase

PHOSPHATE BUFFER SALINE (PBS)

For 1000 ml at pH 7.2-7.4

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Formula</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium chloride</td>
<td>NaCl</td>
<td>8.766gm</td>
</tr>
<tr>
<td>Potassium hydrogen phosphate</td>
<td>KH₂PO₄2H₂O</td>
<td>200mg</td>
</tr>
<tr>
<td>Disodium hydrogen phosphate</td>
<td>Na₂HPO₄2H₂O</td>
<td>1.440gm</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>KCl</td>
<td>200 mg</td>
</tr>
<tr>
<td>Phenol red</td>
<td></td>
<td>2 ml</td>
</tr>
</tbody>
</table>
DEBECO'S MODIFIED EAGLE'S MEDIUM (DMEM)

For 1000 ml

- Measured out 5% less distilled water than desired total volume of medium using a mixing container that is as close to the final volume as possible.
- Added powdered medium at 15 to 30°C (room temperature) and water with gentle stirring.
- Rinsed out inside of package with distilled water to remove all traces of powder.
- Added 3.7gm sodium bicarbonate (NaHCO₃) per liter of DMEM.
- Diluted to a desired volume with distilled water.
- Adjusted pH of medium to 0.2-0.3 below desired final working (pH=7.4) with the use of 1N sodium hydroxide and/or 1N hydrochloric acid is recommended (add slowly with stirring). After pH had been adjusted, the container is kept closed until medium is filtered.
- Sterilized immediately by membrane sterile filtration. Medium was filtered by using a peristaltic pump.
- Penicillin:
  1 lakhs units/litre
  100 units/ml
  1 lakhs units ~ 60mg
- Streptomycin:
  100 mg/litre
  100 μg/ml
- Add HEPES 15mM-25mM (15mM,i.e. 3.57 g/litre)
TRYPSIN EDTA FOR MONOLAYER CELL LINE

- Ethylene diamine tetraacetate (EDTA) : 0.02% w/v
- Trypsin (1:250) : 0.05% w/v

For 500 ml

- Sodium chloride : 4.383gm
- Potassium hydrogen phosphate : 100mg
- Disodium hydrogen phosphate : 720mg
- Potassium chloride : 100mg
- Phenol red : 1 ml
- EDTA : 100mg
- Trypsin : 250mg

pH 7.2 – 7.4

Sterile filtration was performed using (0.22μm) millipore filter by peristaltic pump machine.

FCS DECOMPLEMENTATION

Thaw at room temperature.
Set up water bath at 56°C and put FCS containing bottle in it for 30 minutes.
Stored the aliquotes at -20°C.
METHODOLOGY

CELL CULTURE

The human breast cancer cell line MCF-7 was obtained from national centre for cell science, Pune, India. The cells were maintained in a 5% CO$_2$ humidified atmosphere at 37°C in DMEM supplemented with FBS (10%) and antibiotics (streptomycin 200μg /ml and penicillium 100 units /ml). Cells were harvested from 80% to 90% confluent culture by trypinization.

CYTOTOXICITY OF HYDROXAMIC ACIDS

The antiproliferative effect of N-arylsubstituted hydroxamic acid derivatives against MCF-7 cells were determined using MTT assay in microliter plates. For each experiment, the cells were suspended in DMEM with 10% FBS at 1x10$^5$ / ml and seeded in 96 well plates at 100μl/well and allowed to grow for 24hrs. Each compound initially dissolved in 100% DMSO immediately before use and further into DMEM to obtained ten gradual concentrations ranging from 1.25x10$^{-5}$ to 8x10$^{-8}$M. Each dilution was added to the wells as 100μl/well in quadruplicate. The final concentration of DMSO in the cell culture was 0.5% for the highest drug concentration and in corresponding controls, all others had < 0.25% to avoid the toxicity of DMSO. Hydroxyurea (Aldrich Chemical St. Louis, Mo.) was used as a positive control. Negative control containing only DMSO at identical dilutions was run with each experiment. The plate was incubated in 5%CO$_2$ humidified in atmosphere at 37°C for 48 hrs.
MTT ASSAY

At the end of 48 hrs the viability was evaluated using an assay based on reduction of yellow dye MTT to a purple formazon crystal by the dehydrogenous of the mitochondria, a conversion that occurs only in the living cells. At the end of the treatment intervals, MTT (0.1 mg/well) was added and plate was incubated for 3 more hours. The reduced MTT were solubilized by incubating the cells with 10% sodium dodecyl sulphate (10% w/v in 0.01M HCL) overnight. The absorbance of the solution was measured at 550nm, using a microplate reader (Bio-Tek instruments, USA). All experiments were repeated at least thrice.

RESULTS AND DISCUSSION

Growth inhibition of MCF-7 cells: Dose–Response Comparison.

For the purpose of comparing the MCF-7 growth inhibitions potency of hydroxyurea with that of several structure analogous, compounds 1-19 were studied at concentration of 6.25, 12.5, 25, 50, 75, 100, 150, 200, 300 and 400µM/ml over a 2 days treatment period Figs. 5·2 to 5·20. The differences in potency were maintained at the concentration range from 6.25 to 400 µM/ml.
PLOT OF GROWTH - INHIBITORY DOSE RESPONSE OF HYDROXAMIC ACIDS ON MCF-7 CELLS

![Graph showing the inhibitory dose response of hydroxamic acids on MCF-7 cells.](image)

**FIG. 5.1 HYDROXYUREA**

![Graph showing the inhibitory dose response of N-phenylbenzo hydroxamic acid on MCF-7 cells.](image)

**FIG. 5.2 N-PHENYL BENZO HYDROXAMIC ACID**
PLOT OF GROWTH – INHIBITORY DOSE RESPONSE OF HYDROXAMIC ACIDS ON MCF-7 CELLS

FIG. 5.3 N-PHENYL-4-FLUROBENZOHYDROXAMIC ACID

FIG. 5.4 N-PHENYL-4-NITROBENZOHYDROXAMIC ACID
PLOT OF GROWTH – INHIBITORY DOSE RESPONSE OF HYDROXAMIC ACIDS ON MCF-7 CELLS

FIG. 5.5 N-PHENYL-4-METHY-3-NITROBENZOHYDROXAMIC ACID

FIG. 5.6 N-p-TOLYL-3-NITROBENZOHYDROXAMIC ACID
PLOT OF GROWTH – INHIBITORY DOSE RESPONSE OF HYDROXAMIC ACIDS ON MCF-7 CELLS

FIG. 5.7 N-PHENYL-4-ETHOXYBENZOHYDROXAMIC ACID

FIG. 5.8 N-PHENYL-4-CHLOROBENZOHYDROXAMIC ACID
PLOT OF GROWTH – INHIBITORY DOSE RESPONSE OF HYDROXAMIC ACIDS ON MCF-7 CELLS

FIG. 5.9 N-p-CHLOROPHENYL-4-NITROBENZOHYDROXAMIC ACID

FIG. 5.10 N-PHENYL-2-CHLOROBENZOHYDROXAMIC ACID
PLOT OF GROWTH–INHIBITORY DOSE RESPONSE OF HYDROXAMIC ACIDS ON MCF-7 CELLS

FIG. 5-11 N-m-CHLOROPHENYLBENZOHYDROXAMIC ACID

FIG. 5-12 N-m-CHLOROPHENYL-2-METHOXYBENZOHYDROXAMIC ACID
PLOT OF GROWTH – INHIBITORY DOSE RESPONSE OF HYDROXAMIC ACIDS ON MCF-7 CELLS

FIG. 5.13 N-p-TOLYL-4-ETHOXYBENZOHYDROXAMIC ACID

FIG. 5.14 N-o-TOLYL-4-ETHOXYBENZOHYDROXAMIC ACID
PLOT OF GROWTH – INHIBITORY DOSE RESPONSE OF HYDROXAMIC ACIDS ON MCF-7 CELLS

FIG. 5.15 N-PHENYL-2-IODOBENZOHYDROXAMIC ACID

FIG. 5.16 N-o-TOLYL-2-CHLOROBENZOHYDROXAMIC ACID
PLOT OF GROWTH – INHIBITORY DOSE RESPONSE OF HYDROXAMIC ACIDS ON MCF-7 CELLS

FIG. 5·17 N-o-TOLYL-4-CHLOROBENZOHYDROXAMIC ACID

FIG. 5·18 N-p-CHLOROPHENYL-4-CHLOROBENZOHYDROXAMIC ACID
PLOT OF GROWTH – INHIBITORY DOSE RESPONSE OF HYDROXAMIC ACIDS ON MCF-7 CELLS

FIG. 5.19 N-p-CHLOROPHENYL-4-BROMOBENZOHYDROXAMIC ACID

FIG. 5.20 N-a-TOLYL-2-FUROHYDROXAMIC ACID
TABLE 5.

**IC₅₀ VALUES OF HYDROXAMIC ACIDS**

<table>
<thead>
<tr>
<th>S.No.</th>
<th>HYDROXAMIC ACIDS</th>
<th>IC₅₀(µM)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>N-Phenylbenzo-</td>
<td>96.60</td>
</tr>
<tr>
<td>2</td>
<td>N-Phenyl - 4 - fluorobenzo-</td>
<td>132.26</td>
</tr>
<tr>
<td>3</td>
<td>N-Phenyl - 4 - nitorbenzo-</td>
<td>172.95</td>
</tr>
<tr>
<td>4</td>
<td>N-Phenyl - 4 - methyl-3- nitrobenzo-</td>
<td>178.57</td>
</tr>
<tr>
<td>5</td>
<td>N-p-Tolyl-3- nitrobenzo-</td>
<td>139.19</td>
</tr>
<tr>
<td>6</td>
<td>N-Phenyl - 4 - ethoxybenzo-</td>
<td>183.26</td>
</tr>
<tr>
<td>7</td>
<td>N-Phenyl - 4 - chlorobenzo-</td>
<td>251.47</td>
</tr>
<tr>
<td>8</td>
<td>N-p-Chlorophenyl - 4 - nitrobenzo-</td>
<td>81.11</td>
</tr>
<tr>
<td>9</td>
<td>N-Phenyl - 2 - chlorobenzo-</td>
<td>287.23</td>
</tr>
<tr>
<td>10</td>
<td>N-m- Chlorophenylbenzo-</td>
<td>142.80</td>
</tr>
<tr>
<td>11</td>
<td>N-m- Chlorophenyl - 2- methoxybenzo-</td>
<td>118.62</td>
</tr>
<tr>
<td>12</td>
<td>N-p-Tolyl- 4 - ethoxybenzo-</td>
<td>151.05</td>
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<tr>
<td>13</td>
<td>N-o-Tolyl- 4- ethoxybenzo-</td>
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<td>14</td>
<td>N-Phenyl - 2 - iodobenzo-</td>
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<td>15</td>
<td>N-o-Tolyl- 2- chlorobenzo-</td>
<td>337.55</td>
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<td>N-o-Tolyl- 4- chlorobenzo-</td>
<td>70.93</td>
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<td>17</td>
<td>N-p- Chlorophenyl - 4 - chlorobenzo-</td>
<td>61.99</td>
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<tr>
<td>18</td>
<td>N-p- Chlorophenyl - 4 - bromobenzo-</td>
<td>89.96</td>
</tr>
<tr>
<td>19</td>
<td>N-p-Tolyl- 2-furo-</td>
<td>288.61</td>
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IC₅₀ Value of Hydroxyurea = 307.18µM
CONCLUSIONS

- A total of 18 out of 19 hydroxamic acids had higher inhibitory activities than hydroxyurea (an anticancer drug currently used for the treatment of melanoma, leukemia and ovarian cancer) against MCF-7 cells.

- IC\textsubscript{50} values of hydroxamic acids are found to be in range of 6.2x10\textsuperscript{-5} to 3.38x10\textsuperscript{-4} M.

- Five compounds with IC\textsubscript{50} values in micromolar range are 3- to 5- fold more potent than hydroxyurea (IC\textsubscript{50} = 307.15\mu M). These are, N-p-chlorophenyl-4-chlorobenzo->N-o-tolyl-4-chlorobenzo->N-p-chlorophenyl-4-nitrobenzo- > N-p-chlorophenyl-4-bromobenzo- > N-phenylbenzohydroxamic acids.

- The less potent compound is found to be the N-o-tolyl-2-chlorobenzohydroxamic acid with IC\textsubscript{50} value 337.5\mu M.

- The most potent compound is found to be the N-p-chlorophenyl-4-chlorobenzohydroxamic acid with IC\textsubscript{50} value 61.99\mu M.