CHAPTER 7
ANTITUMORAL ACTIVITY OF SOME LIGAND AND THEIR METAL ION COMPLEXES
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INTRODUCTION

Cancer is a term used for diseases in which abnormal cells divide without control and are able to invade other tissues. Cancer cells can spread to other parts of the body through the blood and lymph systems. More than 100 different types of cancer have been reported [1-3]. Cancers are named for the organ or type of cell in which they start, for example, cancer that begins in the colon is called colon cancer; cancer that begins in melanocytes of the skin is called melanoma. With more than 10 million new cases every year reported, has become most devastating diseases worldwide. The causes and types of cancer vary in different geographical regions, disease burden is immense, not only for affected individuals but also for their relatives and friends [4].

Cancer poses considerable challenges for the health care systems in both poor and rich countries. Much of them suffering, death from cancer could be prevented by efforts to reduce tobacco use, improve diet and physical activity, reduce obesity and expand the use of established screening tests. The American Cancer Society estimates that in 2013 about 174,100 cancer deaths will be caused by tobacco use alone. In addition, approximately one-quarter to one-third of the 1,660,290 cancer cases expected to occur in 2013 can be attributed to poor nutrition, physical inactivity, overweight and obesity [5-10]. Regular use of some established screening
tests can prevent the development of cancer through identification and removal or treatment of premalignant abnormalities; screening tests can also improve survival and decrease mortality by detecting cancer at an early stage when treatment is more effective. “World Cancer Report 2013” provides a unique global view of cancer. It documents the frequency of cancer in different countries and trends in cancer incidence and mortality as well as describing the known causes of human cancer.

The molecular and cellular basis of the multi-step process of malignant transformation is concisely summarized [11-19]. The report contains an overview of cancer prevention, including screening programs for early diagnosis, as well as advances in surgical and medical oncology, including novel drugs targeting tumor-specific signaling pathways. The efforts of the “World
Health Organization” in the fight against cancer are detailed, together with strategies for cancer control. World Cancer Report provides a comprehensive overview of cancer for all health care professionals and the general reader [20-28]. Information is presented concisely, with more than 500 color photographs, diagrams and tables. World Cancer Report also presents opportunities for action at the individual, community and national level. "The global burden of cancer continues to increase. In the year 2000, 5.3 million men and 4.7 million women developed a malignant tumor and 6.2 million died from the disease. The number of new cases is expected to grow by 50% over the next 20 years to reach 15 million by 2020. [29-31].

**Highlights, CPED 2013**

**Tobacco Use**
- Cigarette smoking prevalence in US adults declined between 2005 and 2011 from 20.9% to 19.0%, with significant declines in both men (23.9% to 21.6%) and women (18.1% to 16.5%) as well as in young adults and certain race/ethnic groups (Hispanics and Asians). In addition, heavy smoking declined significantly during this time, reflecting long-term historical trends toward lower cigarette consumption in smokers.
- The high school smoking rate was reduced by 18% from a high of 22.0% to 18.1%, a new low, between 2003-2011.
- Apart from cigarettes, the most commonly used tobacco products among high school students in 2011 were cigars (13.1%) and smokeless tobacco (7.7%).
- Raising cigarette prices by increasing excise taxes reduces tobacco consumption. At present, the average state cigarette excise tax rate is $1.48, with wide variation between states ranging from 17 cents per pack in Missouri to $4.35 per pack in New York.
- During 2011-2012, states have spent less on tobacco prevention (<2% of tobacco-related revenue) than in any period since the Master Settlement Agreement in 1998, despite record high revenues from the settlement and tobacco taxes [32].
- In addition to increasing funding for tobacco prevention programs, states must step up the pace in enacting tobacco tax increases and smoke-free workplace laws, which has slowed in recent years.

**Overweight and Obesity, Physical Activity, and Nutrition**
- Updated in 2012, the American Cancer Society Guidelines on Nutrition and Physical Activity for Cancer Prevention highlight the importance of individual nutritional and physical activity choices for cancer prevention and community efforts to facilitate such choices.
- Currently, an estimated 18.4% of adolescents and 35.7% of adults are obese. Increasing rates of obesity observed since the early 1980s appear to have slowed in the past decade, particularly among
women and girls. Obesity prevalence has increased among men in the past decade and has converged with the rate among women.

- The percentage of US high school students who were obese in 2011 varied widely across states; Colorado had the lowest proportion of obese adolescents (7.3%) and Alabama the highest (17.0%).
- In 2011, the prevalence of obesity exceeded 20% in all states; the state with the highest obesity prevalence was Mississippi (35.0%).

Ultraviolet Radiation and Skin Cancer

Many states are taking action on skin cancer prevention by enacting policies to control the indoor tanning industry. Of these states, seven have stronger policies in place that restrict minors’ (< 18 years olds) access to indoor tanning facilities. This strategy along with school-based sun-safe programs could help reduce future risks of developing skin cancers [33].

HPV Vaccination for Cervical Cancer Prevention

To prevent cervical cancer, vaccination against certain types of human papillomavirus (HPV) is recommended for adolescent girls. The initiation of the HPV vaccination series among US females 13 to 17 years of age increased from 25.0% in 2007 to 53.0% in 2011, with 70.7% of those who initiated completing the entire series. Despite these improvements in the past 6 years, the HPV vaccine coverage among adolescent females lags behind other recommended vaccines.

Cancer Screening

- Mammography usage has not increased since 2000. In 2010, 66.5% of women 40 years of age and older reported getting a mammogram in the past two years. Women who lack health insurance have the lowest use of mammograms (31.5%) within the past two years.
- In 2010, 83.0% of adult women (21-65 years of age) had received a Pap test in the past three years. However, there is persistent underuse of the Pap test among women who are uninsured, recent immigrants, and those with low education.
- In 2010, 59.1% of adults 50 years of age and older reported use of either a fecal occult blood test (FOBT) or an endoscopy within recommended time intervals. However, rates remain substantially lower in uninsured individuals and those with lower socioeconomic status. To date, 28 states and the District of Columbia have passed legislation ensuring coverage for the full range of colorectal cancer screening tests.

The main categories of cancer include:

- **Carcinoma** - cancer that begins in the skin or in tissues that line or cover internal organs.
  
  This is often called epithelial tissue, examples: Bladder, Brain, Breast, Cervical cancer.

- **Sarcoma** - cancer that begins in bone, cartilage, fat, muscle, blood vessels, or other connective or supportive tissue.
- **Leukemia** - cancer that starts in blood-forming tissue such as the bone marrow, causing large numbers of abnormal blood cells to be produced and enter the bloodstream.

![Different Kinds of Cancer](image)

*Fig.2. Types of cancer cell in human*

![Cell progression](image)

*Fig.3. Cell progression towards normal to cancer cell*
- **Lymphoma and Myeloma** - cancers that begin in the cells of the immune system.
- Colorectal, Endometrial, Kidney (Renal), Lung, Melanoma (skin), Ovarian, Pancreatic, Prostate and Thyroid.

**MCF-7 cell line from breast cancer**: The MCF-7 is a breast cancer cell line was isolated in 1970 from 69 years old Caucasian women. MCF-7 is an acronym of Michigan Cancer Foundation-7 referring to institute where the cell line was established. The MCF-7 cell line retains several characteristics of differentiated mammary epithelium including ability to process estradiol via cytoplasmic estrogen receptors and the capability of forming domes.

**Organism** - *Homo sapiens* (human)

**Organ** - Mammary gland; breast

**Tissue** - Epithelial

**Disease** - Adrenoma carcinoma

**Receptors** - Estrogen receptor

Fig.4. Human breast cancer and their progression
EXPERIMENTAL

In vitro MCF-7 cytotoxicity assay

Cell-based assays are often used for screening collections of compounds to determine if the test molecules have effects on cell proliferation or show direct cytotoxic effects that eventually lead to cell death. Cell-based assays also are widely used for measuring receptor binding and a variety of signal transduction events that may involve the expression of genetic reporters, trafficking of cellular components, or monitoring organelle function. Regardless of the type of cell-based assay being used, it is important to know how many viable cells are remaining at the end of the experiment. There are a variety of assay methods that can be used to estimate the number of viable eukaryotic cells. Our of them MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was used to study MCF-7 cell viability.
**MTT Tetrazolium Assay Concept**

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] tetrazolium reduction assay was the first homogeneous cell viability assay developed for a 96-well format that was suitable for high throughput screening (HTS). The MTT tetrazolium assay technology has been widely adopted and remains popular in academic labs as evidenced by thousands of published articles. The MTT substrate is prepared in a physiologically balanced solution, added to cells in culture, usually at a final concentration of 0.2 - 0.5mg/mL, and incubated for 1 to 4 hours. The quantity of formazan (presumably directly proportional to the number of viable cells) is measured by recording changes in absorbance at 570 nm using a plate reading spectrophotometer. A reference wavelength of 630 nm is sometimes used, but not necessary for most assay conditions [34, 35].

Viable cells with active metabolism convert MTT into a purple colored formazan product with an absorbance maximum near 570 nm. The percentage of cell growth inhibition was calculated as follows:

\[
\%\text{Inhibition} = \frac{\text{Mean OD control} - \text{Mean OD test}}{\text{Mean OD control}} \times 100\%
\]

When cells die, they lose the ability to convert MTT into formazan, thus color formation serves as a useful and convenient marker of only the viable cells. The exact cellular mechanism of MTT reduction into formazan is not well understood, but likely involves reaction with NADH or similar reducing molecules that transfer electrons to MTT. Speculation in the early literature involving specific mitochondrial enzymes has led to the assumption mentioned in numerous publications that MTT is measuring mitochondrial activity. The formazan product of the MTT tetrazolium accumulates as an insoluble precipitate inside cells as well as being deposited near
the cell surface and in the culture medium. The formazan must be solubilized prior to recording absorbance readings [36].

A variety of methods have been used to solubilize the formazan product, stabilize the color, avoid evaporation, and reduce interference by phenol red and other culture medium components. Various solubilization methods include using: acidified isopropanol, DMSO, dimethylformamide, SDS, and combinations of detergent and organic solvent. Acidification of the solubilizing solution has the benefit of changing the color of phenol red to yellow color that may have less interference with absorbance readings. The pH of the solubilization solution can be adjusted to provide maximum absorbance if sensitivity is an issue; however, other assay technologies offer much greater sensitivity than MTT. The amount of signal generated is dependent on several parameters which include: the concentration of MTT, the length of the incubation period, the number of viable cells and their metabolic activity. All of these parameters should be considered when optimizing the assay conditions to generate a sufficient amount of product that can be detected above background [37].

The conversion of MTT to formazan by cells in culture is time dependent longer incubation time will result in accumulation of color and increased sensitivity up to a point; however, the incubation time is limited because of the cytotoxic nature of the detection reagents which utilize energy (reducing equivalents such as NADH) from the cell to generate a signal. For cell populations in log phase growth, the amount of formazan product is generally proportional to the number of metabolically active viable cells as demonstrated by the linearity of response in culture conditions that alter the metabolism of the cells will likely affect the rate of MTT reduction into formazan. For example, when adherent cells in culture approach confluence and growth becomes contact inhibited, metabolism may slow down and the amount MTT reduction
per cell will be lower. That situation will lead to a loss of linearity between absorbance and cell number. Other adverse culture conditions such as altered pH or depletion of essential nutrients such as glucose may lead to a change in the ability of cells to reduce MTT [38].

Toxicity of the MTT compound is likely related to the concentration added to cells. Optimizing the concentration may result in lower toxicity. Given the cytotoxic nature of MTT, the assay method must be considered as an endpoint assay. A recent report speculated that formazan crystals contribute to harming cells by puncturing membranes during exocytosis. The observation of extracellular formazan crystals many times the diameter of cells that grow longer over time make it seem unlikely that exocytosis of those large structures was involved [39]. In this section we have reported the breast cancer cell, MCF-7 cell assay of N-4-(dihydroxy)benzamidine ligand and their Co(II), Cr(II) and Zn(II) complexes using MTT.

**MTT Solution preparation [40-41]**

1. Dissolve MTT in Dulbecco’s Phosphate Buffered Saline, pH=7.4 (DPBS) to 5 mg/ml.

2. Filter-sterilize the MTT solution through a 0.2 µM filter into a sterile, light protected container. Store the MTT solution, protected from light, at 4°C for frequent use or at -20°C for long term storage.

**Solubilization**

1. Choose appropriate solvent resistant container and work in a ventilated fume hood.

2. Prepare 40% (vol/vol) dimethylformamide (DMF) in 2% (vol/vol) glacial acetic acid. Add 16% (wt/vol) sodium dodecyl sulfate (SDS) and dissolve adjust pH-4.7.
3. Store at room temperature to avoid precipitation of SDS. If a precipitate forms, warm to 37 °C and mix to solubilize SDS.

**MTT MCF-7 cell Assay Protocol**

1. Prepare cells and test compounds in 96-well plates containing a final volume of 100 µL/well and incubate for desired period of exposure.

2. Add 10 µL MTT Solution per well to achieve a final concentration of 0.45 mg/ml, incubate 1 to 4 hours at 37°C.

3. Add 100 µL solubilization to each well to dissolve formazan crystals mix to ensure complete solubilization record absorbance at 570 nm.

**RESULTS AND DISCUSSION**

The N-4-(dihydroxy)benzamidine (PHB) and their Co(II) Cr(II) and Zn(II) complexes were screened using the colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to test their in vitro cytotoxicity against human breast cancer cell line (MCF-7). Doxorubicin was used as the standard drug. The activity of the tested compounds was estimated percent growth inhibition compared to untreated control cells. It was observed that all the compounds showed moderate to good inhibition activity.

The results were expressed as IC₅₀ (inhibitory concentration 50%), the concentration of the compound which inhibits the tumor cell growth by 50% (Table 1). The ligand PHB showed less antiproliferative activity against human breast cancer cell line MCF-7. This may be occurring due to the low cell permeability. Both Cr(II) and Co(II) complexes were effective at
IC$_{50}$ 6 µg and 4 µg respectively. This may be attributed to the octahedral geometry and coordinated water, was shown to selectively concentrate in tumor tissues which helps in cell permeability. DOX IC$_{50}$ (8 µg). The Zn(II) complex was less effective of the value IC$_{50}$ 15 µg. in addition to their unique role in carbonic anhydrase inhibition.

Table 1. comparative study of inhibition of PHB and their metal ion complexes against human breast cancer cell MCF-7

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC$_{50}$ value (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHB</td>
<td>25</td>
</tr>
<tr>
<td>PHBZn</td>
<td>15</td>
</tr>
<tr>
<td>PHBCr</td>
<td>6</td>
</tr>
<tr>
<td>PHBCo</td>
<td>4</td>
</tr>
<tr>
<td>Doxorubicine</td>
<td>8</td>
</tr>
</tbody>
</table>

**CONCLUSION**

N-4-(dihydroxy)benzamidine and their Co(II), Cr(II) and Zn(II) complexes were evaluated for their anticancer activity, obtained results exhibits good results. It was observed that Co(II) and Cr(II) complexes were very much active against the breast cancer cell line MCF-7. We assume that if much study would emphasize on these complexes may lead as anticancer agents.
Reference


22. Results from the 2010 National Survey on Drug Use and Health: summary of national findings. Rockville, MD: Substance Abuse and Mental Health Services Administration; 2011.


