Genistein induces cell death in human cancer cells in culture through mobilization of endogenous copper
RESULTS-IV

Genistein inhibits growth of MDA-MB-231 and MDA-MB-468 cells:

In order to verify cancer cell growth inhibition by genistein, cells from two breast cancer cell lines MDA-MB-231 and MDA-MB-468 were subjected to treatment with varying concentrations of genistein in a MTT assay. As can be seen from the results given in figure 40 (A&B), genistein caused a clear concentration dependant inhibition of growth in human breast cancer cells. These results are in agreement with earlier studies which have shown genistein to possess anticancer activity against various other cancer cell lines [Bannerjee et al., 2007].

Neocuproine inhibits the anticancer activity of genistein against MDA-MB-231 and MDA-MB-468 cells:

Previously it has been shown that copper chelators neocuproine and bathocuproine are able to inhibit the oxidative breakage of cellular DNA (Chapter II), suggesting the involvement of endogenous copper in the process. In the experiment given in figure 41 (A&B) it has been shown that copper chelator neocuproine is able to protect the cancer cells MDA-MB-231 and MDA-MB-468 against the cytotoxic action of genistein, thus confirming the idea that anticancer mechanism of genistein involves mobilization of endogenous copper.

Genistein induced apoptosis in cancer cells is inhibited by copper chelator but not by iron or zinc chelator:

In order to examine the ability of genistein to induce apoptosis in cancer cells, histone/DNA ELISA assay was performed using MDA-MB-231 and MDA-MB-468 cells. As can be observed from the results given in figure 42 (A&B), exposure of cancer cells to genistein (50 μM) for varying time intervals (24h-96h) leads to a progressive increase in the absorbance at 405 nm. Further the
effect of metal chelators was also tested against the genistein induced apoptosis. As given in figure 42 (A&B) and figure 43, copper chelator neocuproine shows a protective effect to a significant degree whereas such protection was not observed to an appreciable extent when either iron or zinc chelators were used (fig.43).

**Genistein limits the cancer cell proliferation in a clonogenic assay:**

Figure 44 gives the result of a clonogenic assay that was performed to support the above findings which demonstrated that the antiproliferative activity of genistein in cancer cells is mediated by endogenous copper (fig 40). Clonogenic or colony formation assay is an *in vitro* cell survival assay based on the ability of a single cell to grow into a colony. The assay is designed to assess a cell’s ability to grow unattached to a surface and it is a method of choice to determine cell’s reproductive death after treatment with a cytotoxic agent. As shown in figure 44, treatment of MDA-MB-231 cells with genistein resulted in the reduction of anchorage independent colonies. However, the presence of copper chelator neocuproine nullifies the effect of genistein leading to cancer cell survival as the number of colonies was found similar to that of control (in the absence of genistein).
Figure 40: Inhibition of cell growth by genistein in breast cancer cell lines (A) MDA-MB-231 and (B) MDA-MB-468

The effect of genistein on cell growth in (A) MDA-MB-231 and (B) MDA-MB-468 breast cancer cells as detected by MTT assay. The cells were incubated with indicated concentrations of genistein for 96 h. MTT assay was performed as described in "Methods". All results are expressed as percentage of control ± standard deviation of triplicate determinations from three independent experiments. P value < 0.01 when compared to untreated control.
Figure 41: Effect of neocuproine on cell growth inhibition by genistein in breast cancer cell lines (A) MDA-MB-231 and (B) MDA-MB-468

(A)

The effect of copper chelator neocuproine on genistein-induced cell growth inhibition in (A) MDA-MB-231 and (B) MDA-MB-468 breast cancer cells as detected by MTT assay. The cells were incubated with indicated concentrations of genistein alone (■) and in the presence of 50μM neocuproine (●) for 96 h. MTT assay was performed as described in “Methods”. All results are expressed as percentage of control ± standard deviation of triplicate determinations from three independent experiments. P value < 0.01 when compared to control.
Figure 42: Effect of neocuproine on apoptosis induction by genistein in breast cancer cell lines (A) MDA-MB-231 and (B) MDA-MB-468

(A)

The Cell Death Detection ELISA Kit (Roche, Palo Alto, CA) was used to detect apoptosis in (A) MDA-MB-231 and (B) MDA-MB-468 breast cancer cells treated with genistein in the absence and presence of neocuproine as indicated in the figure and described in “Methods”. Values reported are ±SEM of three independent experiments. P< 0.01 was considered significant.
Figure 43: Effect of copper, iron and zinc chelators on apoptosis induction by genistein in breast cancer cell MDA-MB-231

The Cell Death Detection ELISA Kit (Roche, Palo Alto, CA) was used to detect apoptosis in MDA-MB-231 breast cancer cells treated with genistein in the absence and presence of copper chelator neocuproine, iron chelator desferoxamine mesylate and zinc chelator histidine as indicated in the figure and described in “Methods”. Values reported are ±SEM of three independent experiments. P value < 0.01 when compared to control.
Figure 44: Response of breast cancer cells MDA-MB-231 to treatment with genistein in the presence of copper, iron and zinc chelators in a clonogenic assay.

MDA-MB-231 cells (3 x 10^4) were plated in 24-well plates as described in “Method”. Culture was supplemented with different concentrations of genistein with or without metal chelators as indicated in the figure. After appropriate culture time (22 days), colonies (>50 cells) were counted. Experiments were carried out in quadruplicate and mean values are reported.
Based on our studies using human peripheral lymphocytes, in the previous chapters we have proposed a mechanism for the cytotoxic action of isoflavones that involves mobilization of endogenous copper ions and consequent DNA degradation through the generation of ROS. Studies in the present chapter demonstrate that genistein-mediated cell growth inhibition in cancer cells could be reversed by copper chelator neocuproine thus confirming the role of endogenous copper in the cytotoxic action of genistein against cancer cells. We have earlier suggested that the preferential cytotoxicity of plant polyphenols towards cancer cells is explained by the observation made several decades earlier which showed that copper levels in cancer cells are significantly enhanced in various malignancies. Since cancer cells contain elevated levels of copper, they may be more subject to electron transfer with polyphenols (Zheng et al., 2006) to generate reactive oxygen species. Thus, because of higher intracellular copper levels in cancer cells it may be predicted that the cytotoxic concentrations of polyphenols required would be lower in these cells as compared to normal cells. Such lower cytotoxic concentrations of polyphenols against cancer cells have been demonstrated (Chen et al., 1998; Lu et al., 2000). Thus the mechanism proposed by us would be an alternative, non-enzymatic and copper-dependent pathway for the cytotoxic action of these compounds that are capable of mobilizing and reducing endogenous copper. As such this would be independent of Fas and mitochondria mediated programmed cell death. Several studies have indicated that apoptosis induction by several polyphenols and other anti-cancer agents is independent of caspases and mitochondria (Piwocka et al., 1999; Leist and Jaattela, 2001) and is accompanied by an increase in the intracellular levels of ROS (Yoshina et al., 2004; Noda et al., 2007; Heiss et al., 2007). This is also consistent with our hypothesis where we propose that plant polyphenols mobilize chromatin-bound copper which is redox cycled and which in turn leads to the formation of reactive oxygen species.
Based on the work presented in this thesis, I would finally like to conclude that mobilization of nuclear copper by plant polyphenols and the consequent prooxidant action could be one of the important mechanisms for their anticancer and chemopreventive properties. Indeed such a common mechanism better explains the anti-cancer effects of polyphenols with diverse chemical structures as also the preferential cytotoxicity towards cancer cells.