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14. ABSTRACT

The major goal of this Concept Award project is to investigate whether a small molecule inhibitor of Bcl-xL will be able to overcome the resistance of Her-2/neu-(+) breast cancer cells to Herceptin. Bcl-xL inhibitor (-)-gossypol shows potent anti-tumor activity to human breast cancer cell lines with high levels of Bcl-xL, but has only minimal effect on human normal breast epithelial cells with low Bcl-xL. (-)-gossypol potently enhanced growth inhibition and apoptosis induction by doxorubicin and docetaxel, the currently used chemotherapeutic agents for breast cancer. Bcl-xL knockdown by siRNA abolished the tumorigenecity of Her-2(+) MCF-7 cells. (-)-gossypol also shows potent synergism with Herceptin in Her-2(+) breast cancer cells in vitro, appears to support our hypothesis that potent and specific Bcl-xL inhibitor might be able to overcome Herceptin resistance and improve the efficacy of Herceptin therapy. The data support that Bcl-xL plays a critical role in breast cancer initiation, progression and resistance. The study provide us a solid foundation to develop (-)-gossypol as a novel molecular targeted therapy for the treatment of breast cancer with Bcl-xL overexpression. (-)-gossypol is now in Phase I-II clinical trial.

15. Subject Terms

Her-2/neu, Bcl-xL, small molecule inhibitor, Herceptin

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I. Introduction:

The major goal of this Concept Award project is to investigate whether a small molecule inhibitor of Bcl-xL will be able to overcome the Herceptin-resistance of Her-2/neu(+) breast cancer. Our *hypothesis* is that anti-apoptotic molecule Bcl-xL may play a role in Herceptin resistance, and a potent and specific Bcl-xL inhibitor might be able to block or even reverse this resistance, thus improving efficacy of Herceptin therapy. This is based on our basic hypothesis that Bcl-xL is the primary molecular target that mediate the anticancer activity of the small molecule Bcl-xL inhibitor, (-)-gossypol, in human breast cancer cells. Our ultimate goal is to develop (-)-gossypol as a novel molecular targeted therapy for the treatment of breast cancer with Bcl-xL overexpression. In this project, we investigated anti-tumor activity and the mechanism of action of (-)-gossypol in human breast cancer with Bcl-xL overexpression, and investigate the potential synergistic effects of (-)-gossypol in combination with Herceptin therapy.

II. Research progress and key research acomplishments:

This project is one-year Concept Award project. Due to the move of the PI's lab from Department of Internal Medicine to Division of Cancer Biology in Department of Radiation Oncology, a 12-month no-cost extension was requested and approved. During the project period, we carried out the experiments proposed in the Statement of Work. Specifically, we carried out the following studies:

II.1. To analyze the correlation of the expression levels of Bcl-xL and HER2 and response to Herceptin, to assess whether there is any link between Bcl-xL overexpression and Herceptin response.

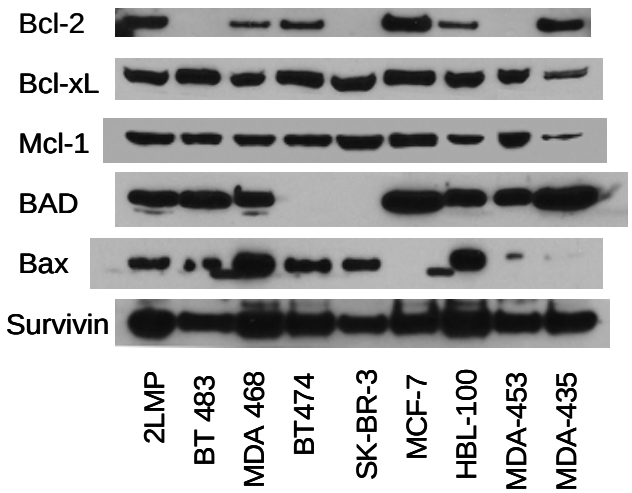
II.1.1. *Using established HER2(+) human breast cancer cell lines with different levels of Bcl-xL, to assess their cellular responses to Herceptin and relation to Bcl-xL expression.*

We examined the Herceptin response of the breast cancer cell lines with Her-2/neu overexpression, i.e., BT-474, SK-BR-3, MDA-453, as well as MCF-7 which has low but detectable Her-2/neu, versus the Her-2/neu-negative MDA-231 cells.

As shown in **Figure 1**, Her-2/neu-positive breast cancer cell line BT-474 has high levels of both Bcl-2 and Bcl-xL, SK-BR-3 has high Bcl-xL but low Bcl-2, MDA-453 has moderate levels of Bcl-xL but high Bcl-2. On the other hand, Her-2/neu-low cell MCF-7 and Her-2/neu-negative cell 2LMP (MDA-231) has high levels of both Bcl-2 and Bcl-xL.

MTT-based cytotoxicity assay shows that BT-474 and SK-BR-3 responds well to Herceptin, while MDA-231 and MCF-7 has no response to Herceptin. (see details in below)

Figure 1. Expression status of Bcl-2 family of proteins in breast cancer cell lines as detected by Western blot.



II.1.2. *Using a Bcl-xL-transfected HER2(+) cell line to see if Bcl-xL overexpression renders the cells more resistant to Herceptin.*

Since we could not find Her-2/neu(+) breast cancer cell line with low Bcl-xL, extensive effort was put into culturing the MCF-7-Her-2 (MCF-7-H18) cells which were transfected with human Her-2/neu gene. As of the end of the project, the MCF-7-H18 cells did not grow well as we had expected. We also obtained a new batch of the MCF-7-H18 cells from Dr. Min Chie Hung's Lab in MD Anderson Cancer Center, and transfected

human Bcl-xL into the MCF-7-H18 cells. However, we were unable to select stable clones for functional assays, due to the fact that both Her-2/neu and Bcl-xL transgenes use the same selectable marker, neo. We have now switched to another selectable marker in our Bcl-xL construct, and will continue this line of investigation together with our newly funded DOD BCRP project.

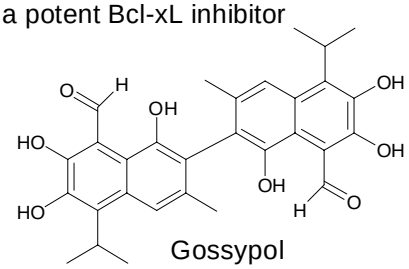
II.2. To investigate the effect of combining Herceptin with potent and specific Bcl-X_L inhibitors, to assess whether functional Bcl-xL inhibition can restore the resistant breast cancer cells' response to Herceptin.

II.2.1. *Using HER2(+) breast cancer cell lines with different levels of Bcl-xL expression to investigate whether Bcl-xL inhibitor can enhance the anti-tumor effect of Herceptin, and restore the resistant cells' response to Herceptin.*

II.2.1.(a) Small molecule inhibitor of Bcl-xL

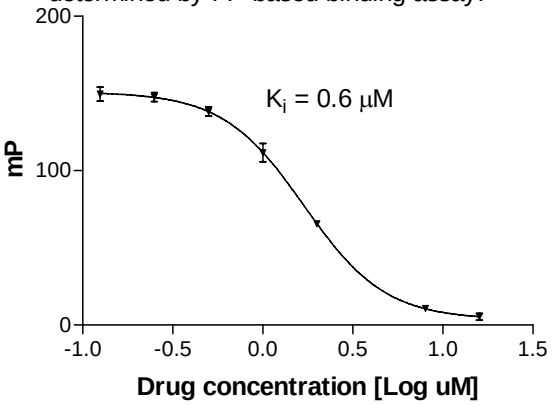
Through structure-based design, we have discovered a natural product from cottonseed, **gossypol**, to be the most potent small molecule inhibitor of Bcl-xL. The chemical structure of gossypol is shown in **Chart I**. Gossypol binds to the BH3 (Bcl-2 homology domain 3) binding pocket of Bcl-xL which is essential for its anti-apoptotic function. Gossypol induces apoptosis in cancer cells with a high level of Bcl-xL protein, but has minimal effect on cancer or normal cells with low Bcl-xL and low Bcl-2. Gossypol has two enantiomer forms, i.e., (-)-gossypol and (+)-gossypol. Previous studies on gossypol for its anticancer activity have been performed exclusively on (±)-gossypol. Our recent data suggest that (-)-gossypol is the active form mediating its anti-tumor activity. In this project, we focused on (-)-gossypol in our studies.

Chart I. Chemical structure of gossypol, a potent Bcl-xL inhibitor



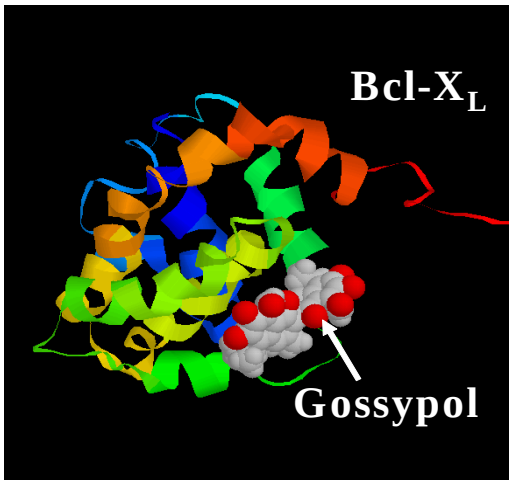
Using a quantitative fluorescence polarization-based (FP) binding assay, we have determined that gossypol potently inhibits the binding of Bcl-xL to Bak BH3 peptide with a K_i value of 0.6 μM, similar to that of Bak protein (**Figure 2**). Gossypol also moderately inhibits the binding of Bcl-2 and Mcl-1 to Bak BH3 peptide with K_i value of <1 μM (data not shown).

Figure 2. Binding of gossypol to Bcl-X_L as determined by FP-based binding assay.



To provide a further insight on the structural basis of the binding between to gossypol and Bcl-xL, we have determined the three-dimensional structure of gossypol in complex with Bcl-xL using multi-dimensional NMR methods. The 3D NMR complex structure conclusively shows that gossypol binds to the BH3 binding site in Bcl-xL, where Bak or Bad BH3 peptide binds (**Figure 3**). Analysis of this 3D structure showed that gossypol optimally interacts with Bcl-xL.

Figure 3. Experimental NMR solution structure of gossypol in complex with Bcl-X_L



II.2.1.(b) Gossypol mediated sensitization of breast cancer cells

To evaluate whether gossypol can enhance the anti-tumor activity of chemo/Herceptin therapy, we employed the Chou-Talalay combination index-isobologram and multiple drug dose-effect analysis method as described[1]. Briefly, in 96-well plates, 3000 - 5000 cells/well human breast cancer cells were treated with (-)-

gossypol and chemo/hormone therapeutic agents, alone or in combination. 4-6 days later, the cell survival was determined by WST-1 cell counting kit (Roche). The combination Index (CI) and Dose Reduction Index (DRI) for each combination dose ratio was calculated using the CalcuSyn software (www.biosoft.com). The optimal dose ratio in the drug combination that gives maximum synergy will be established based upon CI and DRI.[2]

Figure 4 shows the results of gossypol in combination with doxorubicin (DOX) and paclitaxel (Taxol) in MCF-7 cells. The combination index (CI) < 1 indicates a more than additive effect, or synergy, in the drug combination. As shown in Figure 4, gossypol showed synergism in combination with DOX (CI = 0.56) and Taxol (CI = 0.24).

Similar results were also observed in T47D cells. The table 1 summarizes the combination data in both MCF-7 and T47D cells. From Table 1, we conclude that (-)-gossypol have better anti-tumor activity and more synergy than (+)-gossypol, in combination with chemotherapeutic agents, DOX, Taxol and gemcitabine (Gem). Gossypol showed minimal toxicity to MCF-10A cells, the transformed human mammary epithelial cell line which has low levels of Bcl-xL (data not shown).

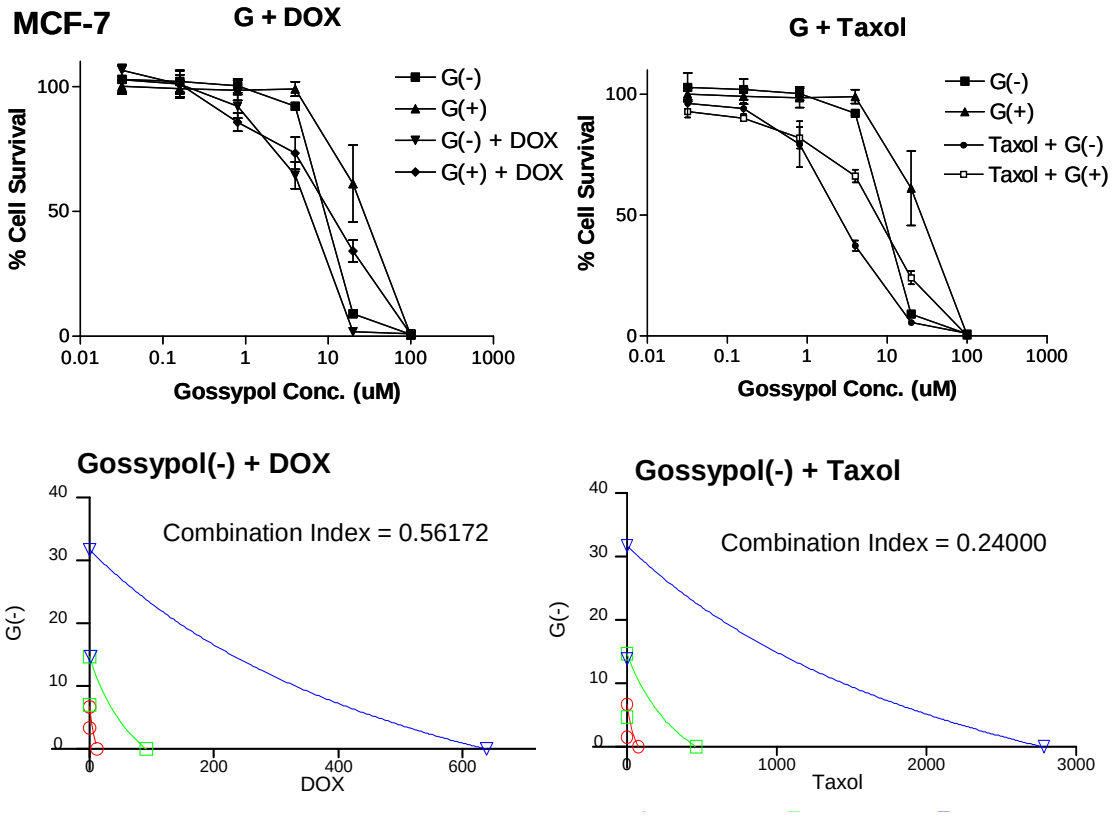


Figure 4. Gossypol enhances cytotoxicity of chemotherapeutic agents in MCF-7 cells. The lower two graphs are isobolograms corresponding to the WST assay data above. (n=3)

Table 1. Summary of drug combination data in MCF-7 and T47D cells.

		IC50 (nM)			**Fold sensitization** (Dose reduction Index)	
		Chemo only	plus G(-)	plus G(+)	G(-)	G(+)
T47D	DOX	75.77	4.2	7.74	18.0	9.78
	Gem	8.601	0.4	9.20	21.3	0.93
	Taxol	2.367	<0.001	0.006	>20	
MCF7	DOX	175.3	<10	97.61	>17.5	1.79
	Gem	54.4	<3	29.11	>18	1.87
	Taxol	7.925	<1	3.33	>8	2.37

II.2.1.(c) Gossypol in combination with Herceptin immunotherapy

Similarly, we examined the combination activity of (-)-gossypol with Herceptin (Genentech, Inc.), in the same manner as described above. Briefly, cells in 96-well plates were treated with gossypol and Herceptin at respective fixed ratios as indicated in the graphs. 4-6-days after treatment, the cell survival was determined by WST-1 cell counting kit (Roche), a MTT-based cytotoxicity assay. The combination Index (CI) and Dose Reduction Index (DRI) for each combination dose ratio was calculated as described above.

Figure 5 shows the representative results of gossypol in combination with Herceptin in Her-2/neu-overexpressing BT-474 breast cancer cells. The combination index (CI) < 1 indicates a more than additive effect, or synergy, in the drug combination. The less of CI and more value of DRI indicate more synergy. As shown in Figure 5, gossypol showed synergism in combination with Herceptin, at ratios of Gossypol:Herceptin = 1:2 (uM:ug/ml) (CI = 0.53), 1:1 (CI = 0.58), 2:1 (CI = 0.28), or 4:1 (CI = 0.22). Therefore, the drug ratios do have a significant impact on the effect of the drug combination. **Figure 6** shows the dose-effect curve and isobologram based on Chou-Talaylay analysis, generated by CalcuSyn.

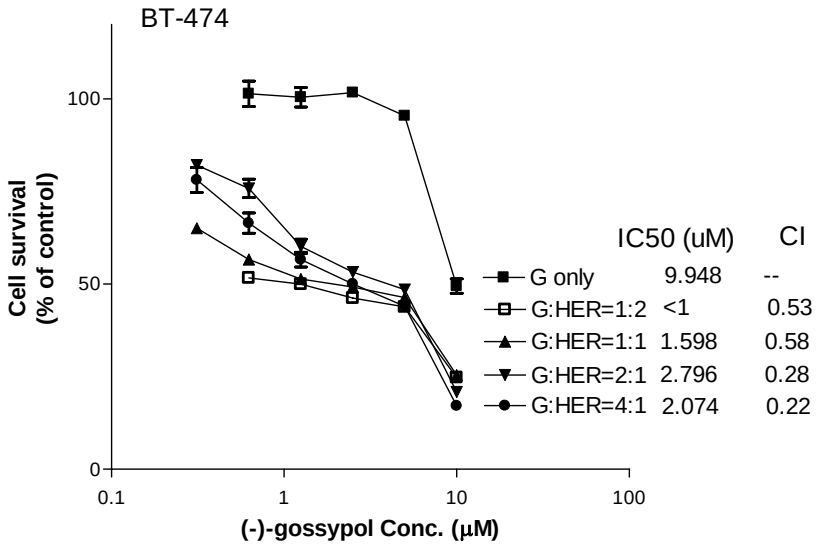


Figure 5. Herceptin enhances cytotoxicity of (-)-gossypol in Her-2/neu-overexpressing BT-474 cells. (n=3)

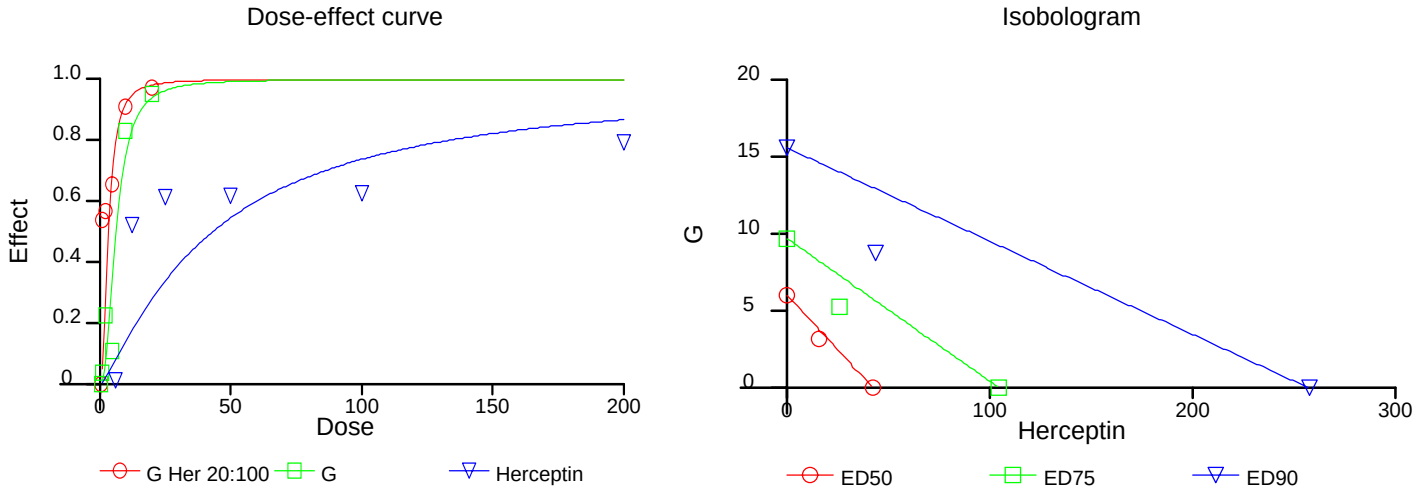


Figure 6. Dose-effect curve and isobologram based on Chou-Talaylay analysis. Combination of Herceptin and (-)-gossypol at the ratios used showed synergy in Her-2/neu-overexpressing BT-474 cells. (n=3)

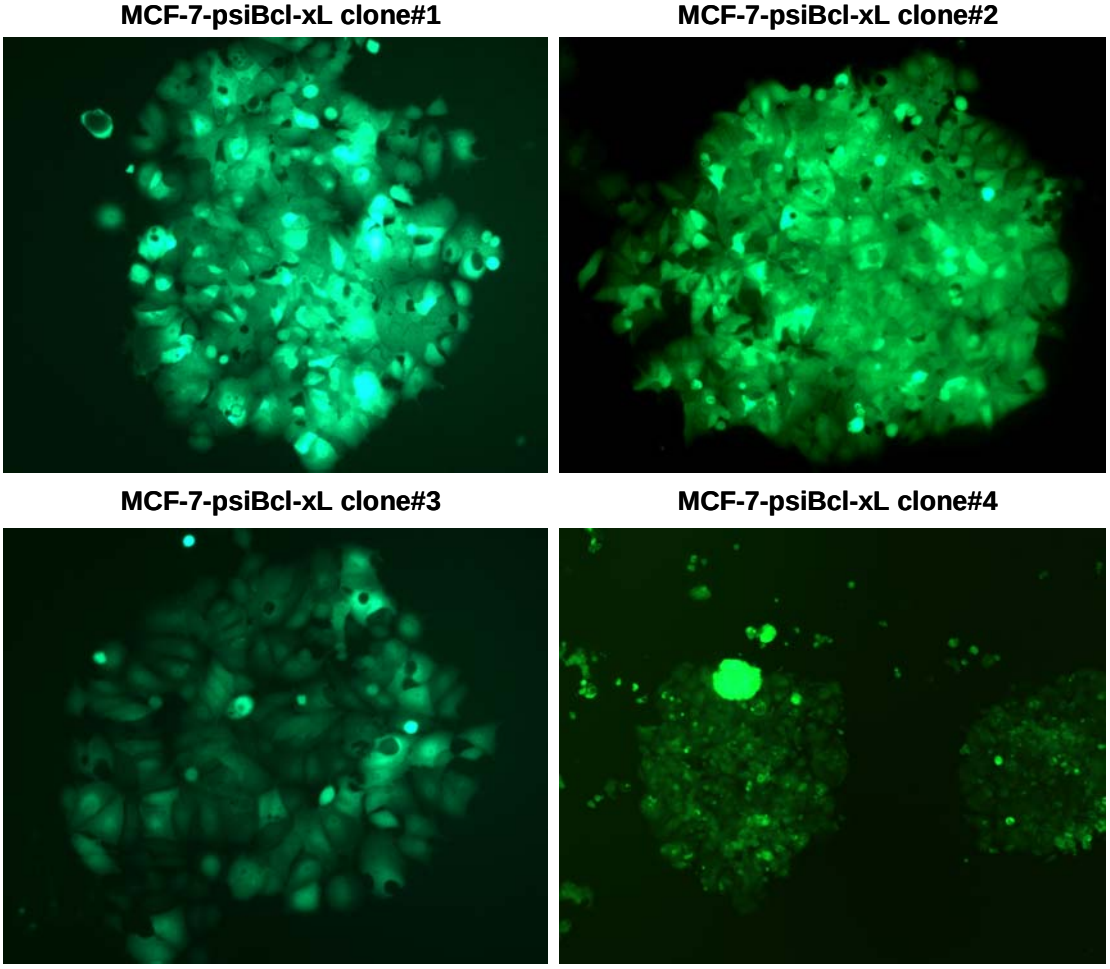
Similarly, SK-BR-3 cells also showed synergism between gossypol and Herceptin, although to a less extent compared with BT-474 (summarized in **Table 2**). These experiments were repeated at least three times with similar results.

Table 2. Summary of gossypol and Herceptin combination in SK-BR-3 cells (at ED75).

Gossypol : Herceptin (uM : ug/ml)	Combination Index (CI)	Dose Reduction Index (DRI)
1 : 2	0.50	1.997
1 : 1	0.44	2.285
2 : 1	0.24	4.185
4 : 1	0.46	2.182

These results demonstrate that, in Her-2/neu-overexpressing breast cancer cells, Herceptin and (-)-gossypol have synergy in inhibiting tumor cell growth. Therefore, the data support that Bcl-2/Bcl-xL inhibitor, (-)-gossypol, might be able to enhance the anti-tumor activity of Herceptin and overcome Herceptin-resistance.

Figure 7. Fluorescent photos of MCF-7 psiBcl-xL stable clones. psiBcl-xL clones show different levels of green fluorescence protein (GFP) expression.



II.2.2. *Small interfering RNA (siRNA) against Bcl-xL*

For molecular target validation of (-)-gossypol, small interfering RNA (siRNA) against Bcl-xL was established. We designed the siRNA based on Bcl-xL mRNA and constructed psiBcl-xL, a vector-based siRNA, for establishing stable cancer cell clones with Bcl-xL knockdown. A siRNA specific to Firefly luciferase gene, psiLuc, was used as vector control.

II.2.2.(a) Transfection of MCF-7 cells with Bcl-xL siRNA vector, psiBcl-xL

MCF-7 cells were transfected with psiBcl-xL as we previously described[3]. In some experiments, FuGene 6 (Roche) was used due to low toxicity and high efficiency for plasmid transfection. Briefly, 60% confluent cells were transfected with 1-3 ug psiBcl-xL or psiLuc DNA each well in a 6-well plate (DNA:FuGene 6 ratio = 1ug:3ul). After 48 hr culture, the cells were collected and lysed for Western analysis,

or trypsinized and replated into 6-well plates, and 50 – 800 ng/ml hygromycin (Invitrogen) was added for stable clone selection.

The transfection efficiency of FuGene 6 for MCF-7 was about 40% - 50%, as evidenced by the green fluorescence in the transfected cells, since psiBcl-xL has green fluorescence protein (GFP) gene to monitor the transfection. MCF-7 cells transfected with psiBcl-xL showed significant cell death (up to 40%).

For stable clone selection, the MCF-7 cells were cultured in hygromycin selection media for two to three weeks and 4 clones with strong green fluorescence were picked. **Figure 7** shows the fluorescent photos of MCF-7-psiBcl-xL stable clones. Note that the different MCF-7-psiBcl-xL clones have different levels of green fluorescence protein (GFP) expression. Theoretically, the level of GFP expression is proportional to the copy number of psiBcl-xL stably transfected in the cells, which is also corresponding to the level of Bcl-xL siRNA transcription from the plasmid psiBcl-xL. Indeed, as shown in **Figure 8**, the Western blot analysis of MCF-7 psiBcl-xL stable clones, the MCF-7-psiBcl-xL clone #1 and #2 showed >95% and >90% down-regulation of Bcl-xL gene expression, respectively, whereas no significant effects on Bcl-xS and Bcl-2. Thus, the clones #1 and #2 have the strongest down-regulation of Bcl-xL, while these two clones also have the strongest green fluorescence. Interestingly, the clone #3 showed >60% down-regulation of Bcl-xL and has moderate green fluorescence, while the clone #4 showed no significant Bcl-xL-downregulation together with poor green fluorescence.

Figure 8. Western blot analysis of MCF-7 psiBcl-xL stable clones. psiBcl-xL clone #1 and #2 showed >95% and >90% down-regulation of Bcl-xL gene expression whereas no significant effects on Bcl-xS and Bcl-2. The clone #3 showed >60% down-regulation.

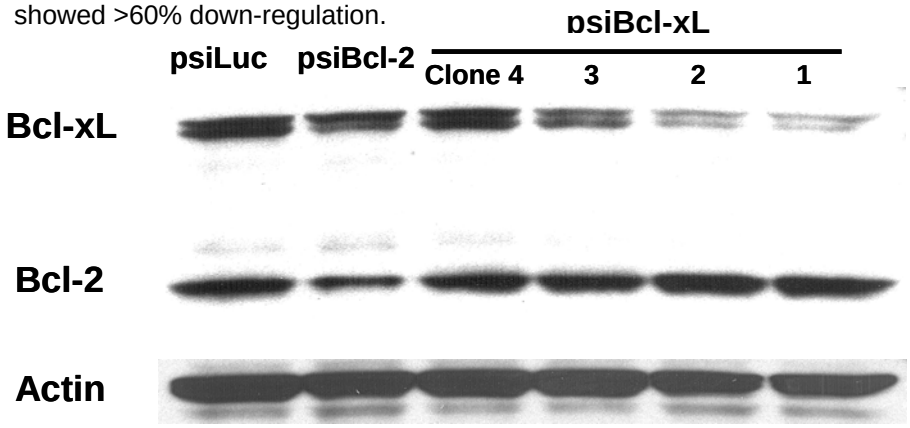
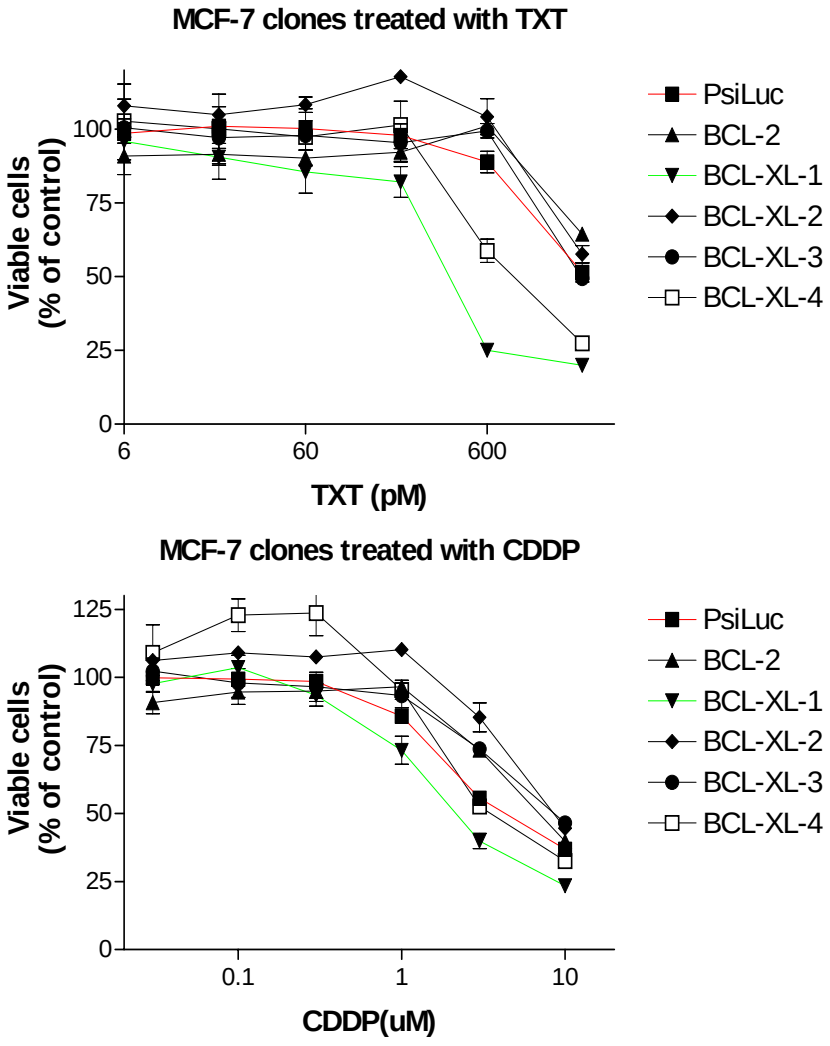


Figure 9. Cytotoxicity assay of MCF-7 psiBcl-xL stable clones. MTT-based WST assay was carried out in 96-well plate. The stable clones of transfected MCF-7 cells were plated 3000 cell/well, treated with CDDP and docetaxel (TXT) for 4 days, add WST-1 as described. The results are plotted as viable cells (% of untreated control).



II.2.2.(b) siRNA-mediated down-regulation of Bcl-xL resulted in sensitization of MCF-7 cells to chemotherapy

Overexpression of anti-apoptotic protein Bcl-xL renders cancer cells more resistance to chemotherapeutic agents. Down-regulation of Bcl-xL by psiBcl-xL will overcome this apoptosis-resistance, thus enhance the induction of cell death by chemotherapy. As shown in **Figure 9**, the MCF-7 psiBcl-xL Clone#1 showed 5-fold

more sensitive than psiLuc clone to docetaxel (TXT)-induced cell death (**Figure 9A**). The Clone#1 also showed a moderate sensitization to CDDP, although to a less extent (**Figure 9B**). The Clone#1 is the clone that has the best Bcl-xL gene knockdown (>95%, **Figure 8**).

In conclusion, transfection of MCF-7 cells with psiBcl-xL resulted in up to 90% -- 95% down-regulation of Bcl-xL protein. MCF-7 cells transfected with psiBcl-xL showed significant increase of cell death and/or apoptosis, slowed cell growth and increased sensitivity to chemotherapeutic agents. This tumor inhibitory activity of psiBcl-xL is related to the down-regulation of the intracellular level of Bcl-xL protein, by specific RNA-interference of Bcl-xL mRNA.

II.2.3. Proof-of-concept pilot in vivo study

II.2.3.(a) Tumorigenecity of MCF-7 stable clones with Bcl-xL knock-down

For each MCF-7-psiBcl-xL stable clones, 10 x 10^7 cells were inoculated in mammary fat pad of ovariectomized female nude mice (Ncr-nu/nu), after E2 pellets were put in. The tumorigenecity of these MCF-7 clones was summarized in **Table 3**. The Clone#1 did not show any sign of xenograft tumor growth 8 weeks after inoculation. The Clone #3 and #4 had only one tumor out of 6 inoculations. Interestingly, Clone #2 had 4 tumors out of 6 inoculations (Table 3), this appears to be consistent with the data shown in **Figure 9** that the Clone #2 cells were more resistant to chemotherapy especially CDDP. This Clone #2 may have other pro-growth/anti-death genes overexpressed to compensate for the down-regulated Bcl-xL for protection. We are currently doing more studies to characterize these clones for their molecular profiles, drug response, etc. These clones provide us with a useful tool to delineate the role of Bcl-xL in ER(+) breast cancer initiation, progression, and drug resistance.

Table 3. Tumorigenecity of MCF-7-psiBcl-xL stable clones in nude mice (Day 19)

Mouse #	psiLuc	psiBcl-xL-1	psiBcl-xL-2	psiBcl-xL-3	psiBcl-xL-4
1	21.44	0	46.4	0	0
2	23.28	0	0	0	0
3	226.48	0	48.75	0	0
4	72.48	0	127.87	13.50	0
5	51.77	0	0	0	46.23
6	0	0	7.7175	0	0
Mean	65.91	0.00	38.46	2.25	7.70
SD	82.66	0	49.12	5.51	18.87

Values are tumor sizes (mm3).

II.2.3.(b) Proof-of-concept pilot in vivo study in Her-2/neu(+) breast cancer animal model, to see whether Bcl-xl inhibitor can enhance or restore the breast cancer response to Herceptin *in vivo*.

We have tried BT-474 and MCF-7-H18 xenograft model, however, both cell lines did not generate reliable xenografts as we have seen in our other breast cancer models. We are still working on the optimal conditions to generate these tumor models, and will carry out in vivo gossypol and Herceptin experiment once these models are ready. These studies will be carried out together with the newly funded DOD BCRP and NIH grants.

III. Reportable outcomes: (One manuscript is in preparation, will be submitted soon)

1. Two peer reviewed grants awarded in 2006:

Based on the data obtained partly from this BRCP Concept grant, we applied and obtained two peer reviewed federal grants:

1 R01 CA121830-01 (Xu) 12/1/06 – 11/28/11
NIH/NCI $250,000
"Tumor-targeted silencing of Bcl-2/Bcl-xL by the self-assembled siRNA-nanovectors"
The major goal of this proposal is to develop the tumor-targeting siRNA-nanovectors as novel molecular therapy targeting Bcl-2/Bcl-xL for human cancers with Bcl-2/Bcl-xL over-expression.
Role: Principal Investigator

BC052329 (PI: Liang Xu) 7/1/06 – 6/30/07
DOD BCRP Concept Award $75,000
"Tumor-targeted silencing of Bcl-2/Bcl-xL by the self-assembled Herceptin-directed siRNA-nanovectors"
The major goal of this proposal is to develop the Herceptin-directed tumor-targeting siRNA-nanovectors as novel molecular therapy targeting Bcl-2/Bcl-xL for human breast cancers with Bcl-2/Bcl-xL over-expression.
Role: Principal Investigator

2. Two abstracts funded from this grant were presented in international and national meetings.

- Liang Xu, et al. Discovery and therapeutic potential of novel Bcl-2/Bcl-xL small-molecule inhibitors in human breast and prostate cancer. *International Conference on Tumor Progression and Therapeutic Resistance.* Philadelphia, PA, November 8-9, 2004. (Dr. Xu was awarded **2nd Prize of Poster Award)**.
- Xu L, et al. Therapeutic potential of Bcl-2/Bcl-xL small-molecule inhibitor in human breast cancer in vitro and in vivo. DOD BCRP Era of Hope 2005 Meeting, Philadelphia, PA, June 8-11, 2005. (Poster P67-19)

3. One investigational new drug (IND) application filed in 2004, on (-)-gossypol Phase I trial in human beings.

Based on the exciting data obtained partly from this BCRP project, the IND for (-)-gossypol was filed in 2004 and approved by FDA in 2005. (-)-gossypol is now in **Phase I clinical trials**. The **Phase II clinical trial** of (-)-gossypol will start soon in University of Michigan, will include breast cancer patients.

4. One US and International Patent application filed in 2005

Xu L, Lippman ME, Liu M. *RNA-based therapeutics targeting Bcl-xL*. Provisional United States Patent Application filed on 12/27/2004. Full US patent application and international PCT filed on 12/27/2005.

IV. Conclusions:

The major goal of this Concept Award project is to investigate whether a small molecule inhibitor of Bcl-xL will be able to overcome the Herceptin-resistance of Her-2/neu(+) breast cancer. We have investigated the *in vitro* anti-tumor activity of (-)-gossypol, a potent small molecule inhibitor of Bcl-xL, and the potential synergistic effects of (-)-gossypol in combination with Herceptin in Her-2/neu-positive breast cancer cell lines. (-)-gossypol showed potent anti-tumor activity to human breast cancer cell lines with high levels of Bcl-xL, but has only minimal effect on human normal breast epithelial cells with low Bcl-xL. (-)-gossypol potently enhanced growth inhibition by doxorubicin and docetaxel, currently used chemotherapeutic agents for breast

cancer, both *in vitro* and *in vivo*. (-)-gossypol also shows potent synergism with Herceptin in Her-2/neu(+) breast cancer cells in vitro, appears to support our hypothesis that potent and specific Bcl-xL inhibitor might be able to overcome Herceptin resistance and improve the efficacy of Herceptin therapy. The data support that Bcl-xL plays a critical role in breast cancer initiation, progression and resistance. The study provide us a solid foundation to develop (-)-gossypol as a novel molecular targeted therapy for the treatment of breast cancer with Bcl-xL overexpression. (-)-gossypol is now in Phase I-II clinical trial.

V. References:

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