# Chapter 12
# Gene Therapy Against HSP90: Glucocorticoid Receptor-Assisted Cancer Treatment

**Susanta Sekhar Adhikari*, Sujan Kumar Mondal*, and Rajkumar Banerjee**

**Abstract** Heat shock protein 90 (HSP90) is an ideal target for cancer because of its involved participation in multiple cancer-implicated pathways that characterize various hallmarks of cancer. Many of the small molecules reported find limited human use because of non-specific collateral damage, and also because of stability & solubility issues. Here we intend to give a different strategy to eliminate HSP90 that too in cancer-specific manner. Towards this we show an interesting property of expressed glucocorticoid receptor (GR) in cancer cells. In these cells GR can be targeted selectively by a newly designed cationic lipid-based liposomal formulation (DX) for selective gene expression. We designed an artificial micro-RNA targeted to certain highly conserved domains in 3′-untranslated region of HSP90, and delivered it as plasmid with DX to different cells and tumor-bearing mouse-models. We observed targeted reduction of HSP90 expression with concomitant reduction of various pro-proliferative, HSP90 client proteins including various kinases and growth factor receptors. Interestingly, anti-apoptotic yet HSP90 client protein, p53 are up-regulated. Together it affected the significant reduction of tumor volume/mass. We hereby describe the development of first 'gene therapy' strategy to target HSP90 by manipulating cancer cell-associated GR, for effective tumor reduction with minimal collateral damage.

**Keywords** Glucocorticoid receptor • Gene delivery • Liposome • Cationic lipid • Dexamethasone • Artificial miRNA • Tumor

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## Abbreviations

| | |
|---|---|
| ADP | Adenosine diphosphate |
| Akt/PKB | Protein kinase B |
| AR | Androgen receptor |
| ATP | Adenosine triphosphate |
| Bcl2 | B-cell lymphoma 2 |
| Cdc37 | Cell division cycle 37 |
| Cdk | Cyclin-dependent kinase |
| Chk1 | Checkpoint kinase 1 |
| CMV | Cytomegalovirus |
| DAPK | Death associated protein kinase |
| Dex | Dexamethasone |
| DODEAC or DO | N,N-di-n-octadecyl-N,N-(2-hydroxyethyl) ammonium chloride |
| DX | Dexamethasone-associated cationic lipid-based liposomal formulation |
| EGFR | Epidermal growth factor receptor |
| ER | Estrogen receptor |
| ErbB2 | V-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2 |
| ERK1 | Extracellular-signal-regulated kinase 1 |
| FGF | Fibroblast growth factors |
| GAPDH | Glyceraldehyde 3-phosphate dehydrogenase |
| GPR30 | G protein-coupled receptor 30 |
| GR | Glucocorticoid receptor |
| GRE | Glucocorticoid responsive elements |
| GSK3β | Glycogen synthase kinase 3 beta |
| HDAC6 | Histone deacetylase 6 |
| Her2 | Human epidermal growth factor receptor 2 |
| HSBP1 | Heat shock factor binding protein 1 |
| HSE | Heat shock element |
| HSF | Heat shock factor |
| HSP90 | Heat shock protein 90 |
| hTERT | Human telomerase reverse transcriptase |
| IFNγ | Interferon gamma |
| IGF1 | Insulin-like growth factor 1 |
| JAK | Janus kinase |
| LBD | Ligand binding domain |
| MAPK | Mitogen-activated protein kinase |
| MDM2 | Mouse double minute 2 homolog |
| miRNA | Micro RNA |
| MRP1 | Multidrug resistance protein 1 |
| mTOR | Mammalian target of rapamycin |
| NFκB | Nuclear factor kappa-light-chain-enhancer of activated B cells |

| | |
|---|---|
| NHR | Nuclear hormone receptor |
| PDK1 | Pyruvate dehydrogenase kinase isoenzyme 1 |
| P-gp | P-glycoprotein |
| Plk | Polo-like kinase |
| qRT-PCR | Quantitative real-time polymerase chain reaction |
| SAR | Structure activity relationship |
| SR | Sigma receptor |
| STAT | Signal transducer and activator of transcription |
| TUNEL | Terminal deoxynucleotidyl transferase dUTP nick-end labeling |
| UTR | Untranslated region |
| VE-cadherin | Vascular endothelial cell-associated cadherin |
| VEGF | Vascular endothelial growth factor |

## 12.1 Introduction

Heat shock proteins (HSP) are the 'Messiah' class of proteins chaperoning ill-folded protein factors. This hence necessitates ubiquitous expression of HSP in all cells. Under stressed conditions such as during acidosis, elevated temperature, hypoxia etc. cells express markedly enhanced HSP, especially HSP90 [1]. This 90 KD or so evolutionarily conserved chaperone protein has many (to date, clearly defined more than 300 or so) client proteins, which essentially needs chaperoning not only in the event of misfolding or mutation but also for their correct, regulated functioning. A comprehensive list of HSP90 client proteins and interactors (i.e., protein factors directly or indirectly interacting with HSP90) can be seen in a compilation [2]. Among these clients there are multiple protein factors including kinases that are directly involved in the maintenance and progression of cancer. Hence, HSP90 is essentially over-expressed to simultaneously chaperone multiple of these factors complicating the overall pathway-scenario in tumor microenvironment. This therefore critically confounds the management of cancer and its related therapy. Optimistically, in return cancer (or in broader sense tumor) shows overwhelming dependency on HSP90. This leads to what is called as 'HSP90 addiction'. As a result HSP90 inhibitors sensitizes cancer cells more than normal cells as HSP90 in cancer cells becomes more sensitive to these inhibitors [3]. Even though the delivery and execution of HSP90 inhibition should be clinical otherwise the attempt to eradicate cancer will be overshadowed by collateral, irreversible side effects. In overall, anticancer therapies aimed to target and inhibit HSP90 gain importance simply because they in turn are expected to target multiple factors implicated in cancer progression.

Among various client proteins of HSP90, glucocorticoid receptor (GR) holds very limited attention when 'cancer' is in perspective. Since, GR is essentially expressed in almost all cells it cannot be a viable target for cancer, at least theoretically. Mechanistically however, in the absence of hormonal or synthetic ligands in cytoplasm, HSP90 firmly holds GR while clutching its ligand-binding

domain (LBD) and strictly maintains the structural integrity of GR-LBD. HSP90 leaves GR only when the correctly fitting GR-ligand snuggles into the LBD, making GR nucleus-bound [4, 5] Ligand-bound GR as dimer 'transcriptionally regulate' the expression of various genes, including those of gluconeogenesis and these genes are regulated by a collection of glucocorticoid responsive elements (GRE) in genome [6, 7]. Gluconeogenesis is a cellular phenomenon predominantly happening in liver cell hepatocytes, which produce glucose from non-carbohydrate precursors and release it in blood stream for other organs' usage. If gluconeogenesis is the only important phenomenon that is associated with GR then one may question why GR is anyway ubiquitously expressed in all cells! The reason for this ubiquitous expression is not clear though. One of the GR synthetic ligands, dexamethasone (Dex) is a well-known anti-inflammatory agent having limited anticancer effects [8, 9]. Since, cancer is an inflammatory disease we believe any therapeutics intended to reverse inflammatory responses may be logically modified to generate novel anticancer therapeutics.

This chapter is intended to focus on how GR can be utilized for the purpose of selectively targeting cancer. We describe an anomalous behavior of cancer cell-expressed GR which is believed to be compounded by a surprisingly 'compromised' HSP90 in cancer cells [10]. The serendipitous discovery enabled us to design an anticancer liposomal delivery system, which could deliver and express in cancer cells a specially designed artificial micro RNA (miRNA) against HSP90 with high selectivity. The target selectivity was so pronounced that besides HSP90, multiple of its pro-proliferative client proteins were degraded rendering tumors to shrink significantly [11]. Although miles-to-go towards honing specificity in the modality but this study clearly documents the first gene therapy attempt against HSP90 and also attempts to shed some light on the role of GR and HSP90 in tumor microenvironment.

## 12.2 HSP90 in the Crossroad for Cancer Progression

It is now well documented that cancer cells need HSP90 in a 'big way' to maintain various illicit, unregulated pathways towards establishing various 'hallmarks' of cancer. Several of the crucial proteins involved in this juggernaut that propels the advancement of cancer are the active client proteins of HSP90. Most of these crucial client proteins acquire mutations, which could have been otherwise detrimental, are eventually not only tolerated but obtain 'gain of function' to drive oncogenesis. This happens only by the active support of HSP90 for which HSP90 needs to be over-expressed to buffer this calamitous situation so that the cancer maintains a pseudo-homeostasis in the body or specifically in the tumor-growing region. HSP90, which comprises less than 2 % of total cellular protein content in normal un-stressed conditions, expresses 3–4 % during stressed condition. Most of these excess HSP90s, now co-engage other co-chaperones, ATPase modulators and other protein accessories to form individual clusters, to interact with different client proteins in

what is called as chaperone cycle [12]. Subsequently, driven by crucially important multiple ATP hydrolyses in the N-terminal domain of HSP90, the regulation of client protein function and their 'turnover' occurs. Oncogenic mutations in client proteins such as various kinases lead to their higher conformational instability and hence require exaggerated chaperonic help from HSP90 to maintain say, increased kinase activity. This way inherent genetic variation/mutation can be buffered by increased level of HSP90 besides chaperoning usual client proteins [13, 14].

There are multiple instances in literature exhibiting that various protein factors involved in maintaining the hallmarks of cancer are actively chaperoned by HSP90 [This has been excellently reviewed recently by [15]]. Briefly, let's see how. Cancer cell proliferating kinases need HSP90 (and also sometimes HSP90's co-chaperone Cdc37) for their activation and stability. Any hostility against HSP90 such as through chemical inhibition renders these kinases unstable, forcibly degraded with suppression of activities. These kinases play important roles in cell cycle regulation and check points & cell division machinery. These are belonging to: (a) Cell Surface Receptor tyrosine kinases (ErbB2/Her2, insulin receptors and insulin-like growth factor I etc.) [16–18]; (b) Src Family tyrosine kinases (Src, Yes, Fes, Fps, Lck etc.) [19–21]; (c) Serine/Threonine kinases such as Raf Family protein kinases (Raf1, B-Raf) [22, 23]; (d) other Serine/Threonine kinases such MAPK-related protein kinases (MAK, MOK, MRK) [24]; (e) Cell cycle and division-related kinases such as Cyclin-dependent kinases (Cdk4 and Cdk6), other mitotic kinases (Plk, Aurora Kinase B, Chk-1) etc. [25–29].

Survival growth factors such as Insulin receptors, IGF-1 receptors, PDK1, Akt/PKB, mTOR etc. are all chaperoned by HSP90 [17, 18, 30–32]. One of the most important Factors involved for DNA damage response and cell apoptosis is p53. 50 % of cancer carries p53 mutation [33]. So, it is evident that 50 % of cancer still carries wild type p53. Then why is it not properly functioning to contain cancer growth? This is simply because wild type p53's activation is predominantly sequestered by many factors, many of them including mdm2 are critically chaperoned by HSP90. Evidences however show that cancer-causing mutated p53s interact with HSP90 persistently more than the corresponding wild types and exhibit higher binding affinities [34, 35]. Clearly, tumor-associated p53-mutants are HSP90 addicted and hence, down-regulation or inhibition of HSP90 will have more adverse effect on 'HSP90-addicted' subtypes and therefore on cancer cell survival. Other than p53, DAPK (death associated protein kinase) is another prominent HSP90 client, serine/threonine kinase protein, which mediates apoptosis in response to interferon-γ (IFN-γ) and hence acts as tumor-suppressor protein. Its expression is anyway lost in multiple tumors, however inhibition of HSP90 is expected to have deteriorating role on DAPK expression [36]. Expectedly, since DAPK expression contradicts the very survival of cancer cells, DAPK's expression unlike that of other pro-cancerous factor should not be HSP90-addicted. Hence, in cancer cells HSP90 inhibition-linked DAPK's degradation is not expected to out-weigh impact of the degradation of pro-cancerous factors.

Further, sex steroid receptors such as estrogen receptors (ER) and androgen receptor (AR) are chaperoned by HSP90 for its steroid hormone binding [5, 37].

This binding leads to subsequent transactivation and gene expression. In breast and prostate the activation of these respective receptors are highly implicated with cancer progression. The limitless replication potential in cancer primarily involves telomerase, a reverse transcriptase, which incessantly helps elongating and maintaining telomeric end of chromosomes in replicating cancer cells. The human catalytic subunit hTERT in telomerase complex is chaperoned by HSP90 and other co-chaperones [38]. HSP90-inhibition eventually leads to proteolysis of hTERT and retards the mechanism of telomeric DNA length maintenance [39, 40].

Towards strengthening the expansive potential of solid tumors angiogenesis plays a very critical role. Neovascularization and potent angiogenic ability of tumor cells determines the rate of tumor progression and metastasis. Vascular endothelial growth factor (VEGF) and fibroblast growth factors (FGF) bind to the respective tyrosine kinase receptors VEGFR1/2 and FGFR3/4 expressed on tumor-associated endothelial cells' surface and stimulate proliferation of endothelial cells and hence new blood vessels. These receptors are client proteins of HSP90 and their stability and functions are dependent on chaperone-function of HSP90 [41, 42]. Hence, HSP90's inhibition leads to reduction in levels of these receptors and thereby angiogenesis and tumor growth [43]. So, plainly speaking, cancer progression-associated HSP90 client proteins enact more crucial role in cancer than other HSP90 client proteins and hence targeted down-regulation or inhibition of HSP90 will significantly impact the overall growth of tumor.

So, aren't there any of the HSP90 client proteins, whose expression and functionality may be utilized for cancer elimination, yet its expression would not be greatly influenced by HSP90-inhibition? Theoretically if it is possible, then one can design novel anticancer therapeutics aimed to inhibit HSP90 targeted via that client protein. In this regard, let us look into one very interesting aspect of HSP90 chaperone property.

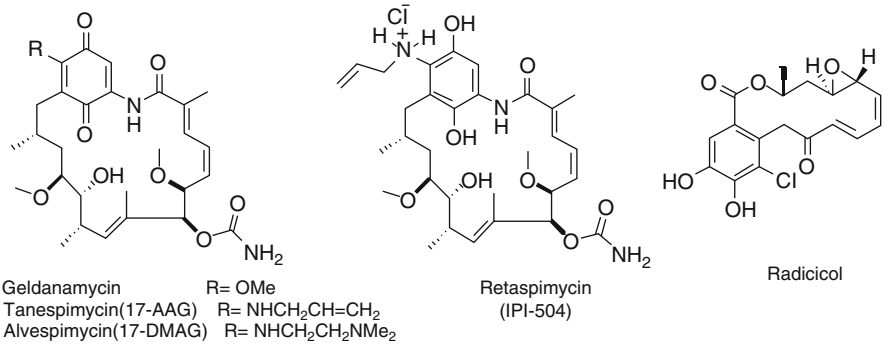
Glucocorticoid receptor (GR) is a client protein of HSP90. The structural integrity of GR-ligand binding domain (GR-LBD) is strictly monitored by HSP90, which allows a space of maximum two molecules of GR-ligand to sit on to LBD [4, 5, 44]. As the ligands bind to LBD, HSP90 release the GR. Ligand-bound GR then localizes in nucleus for further gene transactivation/repression. This interaction of HSP90 with client proteins is functionally related to ATPase cycle involved in the N-terminal domain of HSP90. Generally, binding and hydrolysis of ATP in N-terminal domain of HSP90 precede the release of chaperoned client proteins from it, however with a glaring exception in p53-HSP90 interaction, where ATP binding and not its hydrolysis is all that is needed to release p53 from HSP90 [45, 46]. Hence for GR, ligand binding and ATP hydrolysis in HSP90 seems to occur simultaneously. But most importantly, GR-LBD and HSP90 seemingly share a cellular symbiotic relation. How? It is shown that in one hand, release of GR-LBD by HSP90 involves ATP hydrolysis; on the other hand, GR-LBD stimulates ATPase activity (i.e., which leads to ATP hydrolysis in N-terminal domain) of HSP90 [47]. Conversely, one can hypothesize that the presence of cellular GR (or in other words, GR-LBD) stimulates the ATPase activity and hence influence prompt release of GR as well as other chaperoned client proteins. In cancer cells since chaperoning of HSP90 client

proteins that are implicated broadly with cancer dissemination is rampant, HSP90 needs more and more ATP binding and requires enhanced ATPase activity. When urgency exceeds supply, complete chaos occurs. Towards simultaneous chaperoning of multiple client proteins in cancer cells, HSP90 possibly needs more and more 'illicit' involvement of GR (or GR-LBD or other similar proteins whose identity or functioning are not clear) for strengthening and arming its ATPase activity. Naturally in this scenario, one can expect leniency in strictness about GR-LBD and HSP90 chaperone interaction. The essence of the present chapter is actually based on this hypothesis and an all-important observation related to this forbidden involvement of GR with HSP90, which allowed us to develop a novel anticancer therapeutics.

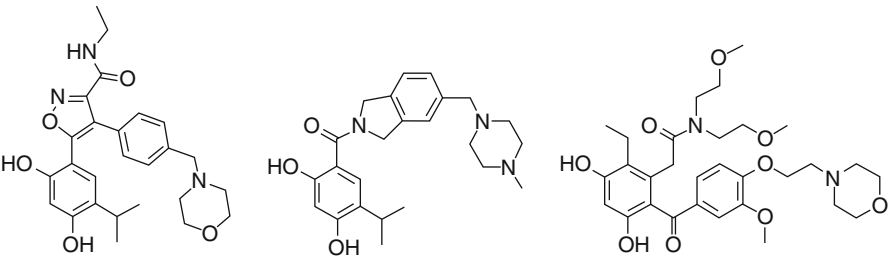
## 12.3 Chemist's Role in Harnessing Hsp90 in Cancer

There has been an upsurge of chemistry-related activities in developing small molecular weight HSP90 inhibitors. These inhibitors are primarily responsible for inhibiting the functional activity of the heat shock proteins. Among heat shock proteins the focus on HSP90 has increased due to its involvement in the several cellular phenomena and more importantly in disease progression. HSP90 keeps the death protein in an apoptosis resistant state by direct association. The wide range of functions of HSP90 results from its ability to chaperone several client proteins that exert a central pathogenic role in human diseases including cancer, neurodegenerative diseases and viral infection. Molecular chaperones are over expressed in wide varieties of cancer cells and in virally transformed cells and are responsible for controlling the activity of signalling proteins. Therefore inhibition of function of these chaperones is essential in controlling cancer cells. The availability of drugs that can specifically target HSP90 and inhibit its function thereby resulting in the depletion of client proteins, has made Hsp90 a novel and exciting target for cancer therapy. This section of the chapter briefly summarizes the current status of both first and second-generation HSP90 inhibitors based on their chemical classification and stage of clinical development.

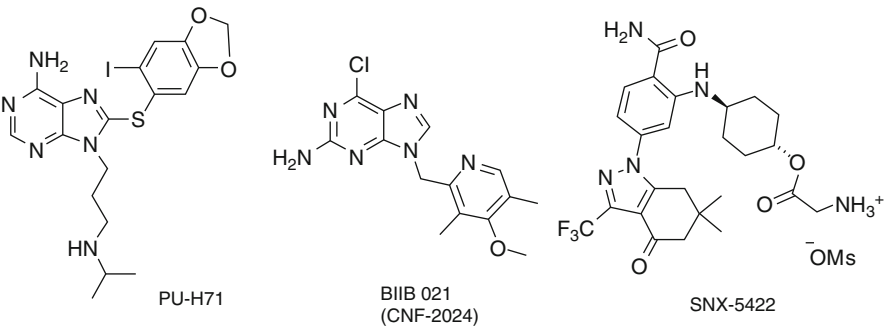
There are now 14 drug candidates that target HSP90 undergoing clinical trials in multiple indications as single agents or combination therapy. These compounds represent a diverse array of chemical matter stemming from natural product scaffolds to synthetic structure-based design. A close inspection of a diverse array of structures that advanced to clinical trials reflects that they can in general be classified according to their similarity to natural product geldanamycin (Scheme 12.I) and the unrelated natural product radicicol (Scheme 12.I) or to the resorcinol- (Scheme 12.II) and purine-scaffolds (Scheme 12.III). Only SNX-5422 falls outside any of these designations (Scheme 12.III). The ansamycins (geldanamycin and herbimycin) and the unrelated natural product radicicol were found to bind to the N-terminal pocket of HSP90 and inhibit its function. The discovery of HSP90 as the target of anticancer activity of geldanamycin has resulted in intense efforts from industry and academia to develop clinically viable small molecule HSP90 inhibitors [48].




**Scheme 12.I** Chemical structures of HSP90 inhibitor geldanamycin and its derivatives and natural product radicicol




**Scheme 12.II** Chemical structures of resorcinol-containing synthetic inhibitors




**Scheme 12.III** Chemical structures of purine and pyrazole-based synthetic inhibitors

## 12.4 Scheme I

Geldanamycin has interesting anti-tumor properties in vivo in animal models. However, issues like stability and hepatic toxicity impeded the translation of this compound in clinics. In an effort to improve tolerability and to address formulation issues, a number of semi-synthetic geldanamycin derivatives have been developed, including tanespimycin (17-AAG), alvespimycin (17-DMAG) and retaspimycin (IPI-504) as depicted in Scheme 12.I. Tanespimycin (17-allylamino-17-desmethoxygeldanamycin, 17-AAG), the first-in-class inhibitor of HSP90, derived from substitution of C-17 methoxy group of geldanamycin with an allylamino group to enter the clinic in 1999 is now in Phase II trials [49]. Unfortunately, 17-AAG is insoluble, difficult to formulate and lacks oral bioavailability. Alvespimycin (17-desmethoxy-17-N,N-dimethylaminoethylaminogeldanamycin, 17-DMAG), obtained from the substitution of the C-17 methoxy group of geldanamycin with N,N-dimethylaminoethylamine, was proved to be potent and to have improved water solubility. The presence of an ionisable amino group is responsible for its increased water solubility, better oral bioavailability and equal or greater anti-tumor activity compared to 17-AAG. This drug has entered Phase I clinical trials in various types of lukemia and solid tumors, and has demonstrated tolerable toxicity [50].

## 12.5 Scheme II

Retaspimycin (17-allylamino-17-desmethoxygeldanamycin hydroquinone hydrochloride, IPI-504), a 17-AAG analogue containing a reduced hydroquinone, prepared by reduction of 17-AAG with sodium dithionite followed by conversion to its hydrochloride salt, has improved water solubility properties thereby facilitating formulation for parental administration. However, hepatotoxicity problem has been observed as similar to 17-AAG and 17-DMAG. Geldanamycin-derived products are also being clinically tested in combination with other chemotherapeutic agents. Imatinib (STI-571, Gleevec) is an effective therapy for only chronic myeloid leukemia characterized by the expression of the oncoprotein, Bcr-Abl. Imatinib inhibits this active tyrosine kinase that renders the cancer cells resistant to apoptosis. Since Bcr-Abl is a Hsp90 client protein, a combination of 17-AAG with imatinib is being tested in Phase I clinical trials in Bcr-AbI positive leukemia with encouraging results [51]. Enhancement of 17-AAG activities has also been reported for proteasome inhibitors such as bortezomib [52]. This effect might result from the increase in protein misfolding induced by 17-AAG, together with the impaired clearance of proteins by the ubiquitin proteasome pathway [53].

A combination of 17-DMAG with arsenic trioxide has emerged as a promising therapeutic combination since they synergize to induce apoptosis and mitotic arrest in leukemic cells.

The natural product radicicol (Scheme 12.I), a macrocyclic antifungal antibiotic was found to bind to the N-terminal pocket of Hsp90 and inhibit its function. Radicicol displays potent *in vitro* activity but was largely inactive in tumor xenograft models probably because of its multiple electrophilic sites such as the strained epoxide and conjugated dienophiles that lead to metabolic deactivation in vivo [54]. However, this scaffold has led to rationally design resorcinol-containing synthetic inhibitors with improved solubility and in vivo potencies. The most advanced clinical compounds in this class are NVP-AUY922 (VER-52296), KW-2478, and AT13387 as well as STA-9090 (Scheme 12.II). STA-9090 is an unspecified novel resorcinol based triazole inhibitor advanced to multiple clinical trials in both advanced solid tumors and hematological malignancies.

## 12.6 Scheme III

A rational choice for HSP90 inhibitors targeting ATPase activity is purine-based compounds (Scheme 12.III). The first synthetic class of such scaffolds was the PU series, such as PU-H71 and PU-D28. These purine-based molecules were developed based on available X-ray crystal structures of HSP90 bound to ATP/ADP as well as to natural inhibitor geldanamycin and radicicol. These PU series molecules mimic the conformation of ADP in the pocket and have a higher affinity for HSP90 than ADP [55]. These molecules possess good solubility and permeability. CNF-2024 (BIIB021), a purine-scaffold induces Hodgkin's lymphoma cell death through inhibition of NF-kB signaling pathway [56] and is orally bioavailable. Interestingly, CNF-2024 was active in cancer models in which 17-AAG and other ansamycin derivatives were inefficient because of the expression by the tumor cells of multidrug resistant proteins (such as, P-gp, MRP-1) [57].

Analysis by computational chemistry and X-ray crystallography of selected ATP-binding proteins allowed the discovery of an orally bioavailable and effective pro-drug, 2-aminobenzamide derivative, SNX-5422, a potent Hsp90 inhibitor which is now in multiple Phase 1 clinical trials [58].

## 12.7 Synthetic Lipids, Liposomes and Cancer Targeting

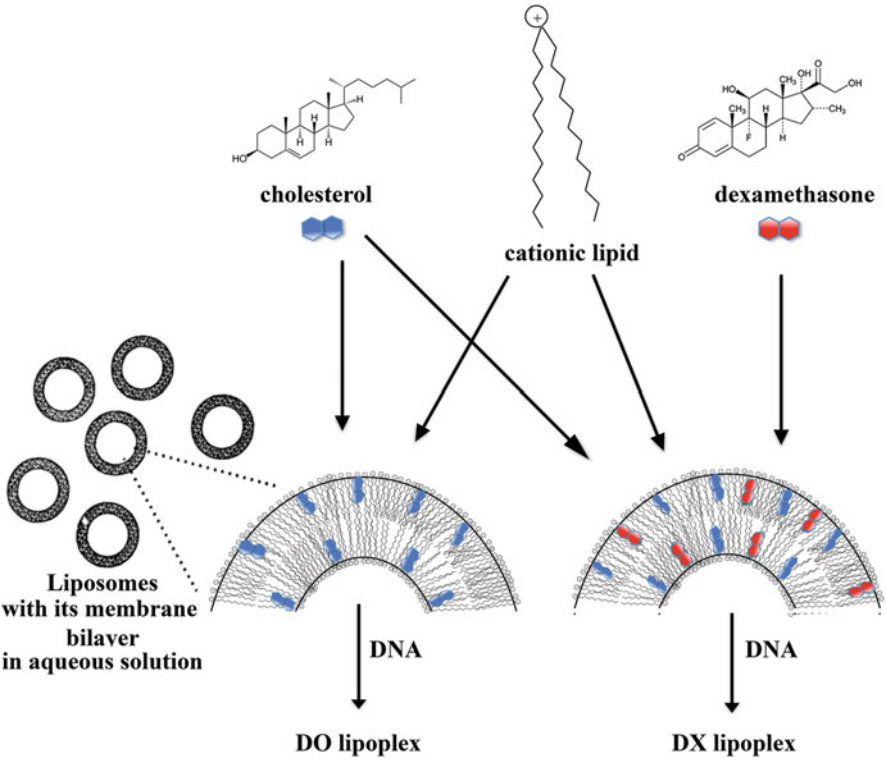
In this section, we would like to discuss in brief the status of the development of cationic lipids, their liposomal behavior and targeted gene delivery aspect. This is important to know to understand the basis of our present discussion, which relates to a new liposomal formulation-based anticancer therapeutics. We will also touch upon how select analogues of these cationic lipids if typically modified develop

into a novel class of selective anticancer molecules. Although under thorough investigation but we will discuss in brief that few of these molecules exhibit an interesting property of regulating HSP90 expression.

### *12.7.1 Synthetic Lipids and Liposomes*

Any amphipathic molecule possessing typical hydrophilic/lipophilic balance (HLB) tends to make aggregates of different interesting characters in aqueous solutions. Typically, an aggregate forming molecule should contain at least a lipophilic moiety (such as a carbon chain length of varied size) and a hydrophilic functional 'head' group (such as an ionic or proton-carrying polar groups etc.). As an example, an ionic molecule conjugated with a single lipophilic carbon chain in aqueous solution can form aggregates called 'micelle' with a hydrophobic/lipophilic core. The same molecule aggregates to form 'reverse micelle' in organic solvent maintaining a hydrophilic pocket in the inside core. However, if instead of one, two lipophilic carbon chains are conjugated to ionic molecules with hydrophilic head group and suspended in aqueous solution, it forms bi-layered aggregates called 'liposomes'. Liposomes (or lipid based compartments) can be classically envisioned as stable lipophilic bubbles, which individually maintain an aqueous pocket [Please see Scheme 12.IV]. Because of this basic structural feature liposomes find many uses especially in pharmaceutical formulations, which can carry hydrophilic drugs in the aqueous pocket and lipophilic drugs in lipid bilayer. If the constituent lipid moiety of liposome carries a positive charge in the hydrophilic head-group region then the liposome 'cooperatively' assumes net positive surface charge. The lipid is called as a cationic lipid and therefore the liposome is called as cationic liposome. Hence, in addition to carrying drugs of different solubility properties, the cationic liposomal formulation bearing net surface positive charge can electrostatically complex with cargoes possessing net negative charges. DNA is a classic example that, because of carrying negatively charged phosphodiester bond among linking nucleotides, can easily complex with cationic liposomes [Scheme 12.IV]. This electrostatic complex is called lipid/DNA complex or 'lipoplex'.

Now, cellular surfaces are negatively charged because of negatively charged membrane-associated components such as proteoglycans, phosphonated fatty lipids, etc. Hence, for DNA the cellular entry is not easy. A lipoplex with net positive charge will have electrostatically more favorable disposition to enter cells, thereby allowing easy cellular entry of DNA cargo. Lipoplex upon treatment to cells are endocytosed and the lipid and the cargo are generally known to dissociate allowing DNA to enter nucleus for further gene expression. The expression of delivered gene following the whole sequential episodes of cellular entry, endocytosis, lipid-DNA dissociation and nuclear entry is collectively called as gene transfection. However, not all cationic lipids have the ability to induce efficient gene transfection. Huge body of evidences available in literature showcasing the structure activity relationship (SAR) study as to how varying functional groups in cationic lipids




**Scheme 12.IV** Schematic representation to show how targeted (DX, i.e., with dexamethasone) and non-targeted (DO, i.e., without dexamethasone) liposomes were formulated. Please note that liposomes acquire a bilayer-membrane in aqueous solution. A cross-section of this bilayer is depicted wherein different components such as cationic lipids and other steroidal co-lipids such as cholesterol and GR-ligand (here dexamethasone) are mutually and cooperatively associated to form different liposomes. Liposomes (with net positive charges) are then complexed with DNA (with negative charges) to form lipoplexes (i.e., lipid-DNA complexes)

affect lipids' gene transfection abilities. For details regarding liposome formation procedure, DNA complexation and delivery, pathways of gene transfection, details of SAR studies, please see representative reviews [59, 60].

Further to this, researchers have developed wide arrays of liposome-based drug/gene delivery reagents, which are target specific for many diseases including cancer [Please see representative, recent reviews such as, [61–63])]. In this context, we have also developed few cancer targeted, cationic lipid-based gene delivery systems. One of the delivery system was ornamented with a hanging ligand targeted to sigma receptor. This receptor is over-expressed on various cancer cell surfaces [64]. Towards targeting estrogen receptor (ER), the highly breast cancer-implicated receptor, we developed cationic lipid formulations with hanging estrogen ligand on liposome surface [65]. Estrogen receptor is a nuclear hormone receptor (NHR)

and is cytoplasmic (and also nuclear but not membrane-associated) in nature. But cells expressing ER also bear membrane-associated ER-like receptors (such as GPR30), which recognize ER-ligands and may also recognize ER-ligand-associated delivery systems. However, glucocorticoid receptor, another NHR, is predominantly cytoplasmic in nature in the absence of its ligand and its endogenous ligands such as hydrocortisone are believed to enter cells through passive diffusion. Moreover, GR is known to express in almost all cells irrespective of their origin and lineage and till now there is no evidence to suggest that there is any membrane-associated receptor that recognizes GR-ligand for cellular uptake. This hence makes GR a cellular target of subdued prominence for the fraternity that develops 'targeted delivery system', especially for cancer.

### *12.7.2 Cationic Lipid-Conjugated Small Molecule as Anticancer Entity*

In this we will briefly discuss how liposome-forming cationic lipids (i.e., lipids of twin aliphatic carbon chain) of a range of specific length if conjugated to various pharmaceutically important molecules, makes a novel class of potent anticancer entities. Firstly, we conjugated a C-8 (i.e., eight aliphatic carbon chain) cationic lipid to 17β-estradiol (ES) at its 17-position using suitable spacer and formed a molecule called 'ESC8'. This molecule besides exhibiting potent killing of ER+ breast cancer cells also exhibited simultaneous autophagy and apoptosis through inhibition of mTOR kinase activity in triple negative breast cancer cells and in tumor xenograft [66]. This molecule shows potentiality to target and treat all stages of breast cancer. Additionally, C-10 analogue of ES (ESC10) showed potent anticancer activity against even ER+ melanoma and pancreatic cancer cells and tumor model [67]. Secondly, upon conjugating a C8-cationic lipid to haloperidol, an anti-psychotic drug and a potent sigma receptor ligand, we developed another anticancer agent (HPC8) that targeted sigma receptor over-expressing cancer cells of varied origin and elicit potent toxicity through Akt downregulation [68]. Thirdly, we once again showed that upon conjugating C8-cationic lipid with GR synthetic ligand, dexamethasone we could develop another anticancer agent (DX8) that acts through down-regulation of JAK/STAT3 pathway [69]. Others have also used the same concept to develop respective novel classes of anticancer agents [70, 71]. Incidentally, we found a rather interesting observation albeit preliminary, with cationic lipid analogues of dexamethasone upon treating to cancer cells. In cancer cells, we found that DX8 transactivates GR many folds more than even dexamethasone (Unpublished data). But more interestingly, DX8-treatment led to selective down-regulation of HSP90 expression in cancer cells, whereas naked dexamethasone visibly increased the expression (Fig. 12.1). This assumes more interest when it is revealed that this molecule synergizes anticancer effect of inhibitors of JAK/STAT3 pathway (unpublished data). Taken together the data indicate that expressed GR in cancer cells can possibly be targeted by modified GR-ligands to regulate HSP90.

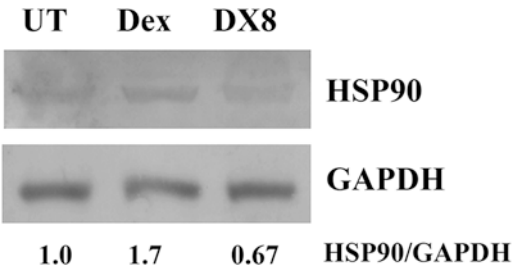



**Fig. 12.1** Cationic lipid-conjugated Dex potentially regulates the expression of HSP90. B16F10 murine melanoma cells were first treated with free Dex or DX8 (this is a new derivative of Dex formed by conjugating a C8 chain length cationic lipid; for details of this structure please see Ref. [69]) or kept untreated (UT) for 24 h. Following this the cells were harvested and Western blot analysis was done on the respective cellular lysates. The level of expression of HSP90 was compared to that of GAPDH

This might lead to selective anticancer effect. Beyond this preliminary data we will now explain how a different modality of GR-targeting can elicit selective regulation of HSP90 in cancer cells.

## 12.8 Glucocorticoid Receptor (GR) as an Under-Utilized Cellular Target in Cancer

GR is a ubiquitously expressed nuclear hormone receptor residing in the cytoplasm as hormone-unbound state. It is expressed in most cells if not all, including cancer and non-cancer cells. Upon ligand (or glucocorticoid) binding GRα isoform translocates itself to nucleus for classical gene transactivation. The gene transactivation includes genes related to maintenance of glucose homeostasis and gluconeogenesis, anti-inflammation, immunosuppression etc. Among various species, the sequence homology of GR DNA binding domain (DBD) and its LBD is highly conserved. Obviously, among the same species sequence homology of GR-DBD and LBD do not vary among cells of normal and cancer lineage. As a result there is no evidence exhibiting differential affinity of GR-LBD to glucocorticoids in normal and cancer cells. Hence, in the context of selective targeting of cancer cells over normal cells, using GR as the cellular target and glucocorticoids as targeting ligand, one should become naturally skeptical as to how to obtain selectivity. Moreover, since GR is a cytoplasmic receptor and does not express on membrane surface it is rather difficult to design therapeutics, which could target any cell of interest expressing GR. There is also no universal evidence to believe that unlike other NHRs such as estrogen receptors (ER), or cell surface receptors such as sigma receptors (SR), GR is selectively over-expressed in cancer cells. For these legitimate reasons, among the fraternity who develops anticancer therapeutics, GR-targeting never received any attention.

## 12.9 GR-Mediated Gene Delivery: A Cancer Selective Phenomenon

We have shown previously that a surface-functionalized liposomal gene delivery system can target cancer cells by the virtue of over-expressing sigma receptors on cell membrane for selective gene expression [64]. We have also shown that using estrogen as ligand for a liposomal gene delivery system one can target ER-expressing breast cancer cells, although ER is a cytoplasmic receptor, for selective gene expression [65]. This happened because of targeting possibly via ER-like membrane receptors such as G-protein coupled receptor (GPR-30), which has high affinity for estrogens. Clearly, there existed helping factors, which helped selective uptake of these receptor-targeting liposomal gene delivery systems in cancer cells. No evidence is present in literature to believe that GR also has a GR-like membrane receptor, which will work in mutual harmony to pull in glucocorticoids or GR-ligands inside cells only to be attracted to LBD of GR residing in cytoplasm. To our knowledge, there is single evidence, wherein GR is over-expressed during phenotypic change from adenoma to carcinoma in adrenocortical tumor [72]. Moreover, there is an indication that among prostate cells all the cellular phenotypes including normal to aggressive cancer cells of different stages expresses GR. There is an exception to only one stage of prostate cancer (the cells of which is identified and preserved as LNCaP cells), where no GR expression is visible [9]. Barring these examples, it is unquestionably established that GR is ubiquitously expressed in most cells, generally with basal levels. Hence to begin with, there is no reason to believe that a GR-targeted delivery system could maintain cancer cell-selective delivery property because of GR's ubiquitous presence.

Initially, we intended to develop a general transfection agent, which could deliver genes in all cell types. Towards this, we hypothesized that GR could be used as the potential cellular target because of its ubiquitous presence. But we had no answer to the primary question as to how GR, which is not a cellular membrane but a cytoplasmic protein could be targeted? Do natural glucocorticoids enter cells only by passive diffusion or do they enter by attaching to some hypothetical GR-like membrane receptors, whose identity is yet to be known? We hence questioned that if this delivery agent is successfully developed as a GR-assisted, general transfection agent could this be used as a probe-formulation to discover those hypothetical GR-like membrane receptors (if any)?

Based on these 'intangible' notions we first developed a potential GR-targeted cationic lipid-based gene transfection formulation. Any cationic lipid-based gene transfection agent uses a cationic lipid and a co-lipid, such as cholesterol, DOPE etc. Cholesterol (Chol) lends stability to the liposomal formulation [73]. We have used Chol along with our previously developed cationic liposomal formulations for efficient gene transfection [74, 75]. We hypothesized that if we simply mix dexamethasone (Dex), a synthetic & steroidal GR-ligand with a closely similar structure as cholesterol, in the formulation we may achieve potent GR-targeting.

Because of structural similarity Dex may co-adjust with Chol in the lipid bilayer without compromising the stability and gene delivery ability of the formulation. Soon, we indeed found this to be true [Scheme 12.IV].

## 12.10 Scheme IV

The Dex containing formulation is also called as DX or 'targeted formulation'. We also made a 'non-targeted', Dex-less formulation called DO. Please see the representation of the formulations in Scheme 12.IV. The formulations bearing net positive charge are electrostatically complexed with negatively charged DNA and they are respectively named as 'DX' or 'DO' lipid-DNA complexes (or, lipoplexes). When DX lipoplex (i.e., which contains Dex) is treated to cancer and non-cancer cells we find the following interesting observations:

(a) DX transfects cancer cells much more efficiently than it does in non-cancer cells.
(b) DX-mediated transfection in cancer cells is significantly inhibited following GR antagonism and GR-siRNA-mediated GR down-regulation.
(c) No such inhibition of DX-mediated transfection is observed in GR-down-regulated/inhibited non-cancer cells.
(d) We observed simultaneous nuclear translocation of GR and DX in cancer cells, but it is not observed in non-cancer cells. No such effects were observed following DO-mediated transfection in either cancer or non-cancer cells (Fig. 12.2a).

Please see Mukherjee et al. [10] for the wholesome data as discussed above.

The observations as delineated above unraveled some invaluable points, which we believe is the mainstay of the current chapter. These observations may be interpreted as following:

(a) DX lipoplex could transfect cancer cells in a GR-selective manner.
(b) In cancer cells, DX lipoplex could recognize GR-LBD, as a result GR was activated and translocated to nucleus.
(c) In all probability, the GR-activation was performed by intact fraction of DX lipoplex, which continued to carry lipid and co-lipid components besides Dex.
(d) This means that GR-LBD in cancer cell-associated GR could not differentiate between 'Dex' molecule and DX lipoplex and allowed DX to bind to it. This led to nuclear localization of DX components that include cationic lipids.
(e) Non-cancer cells did not exhibit similar nuclear localization of DX. This indicates that GR-LBD in non-cancer cell-associated GR could not recognize DX.

To answer, why non-cancer cells' GR could not recognize DX, one can take in consideration the role of chaperone protein HSP90 in maintaining the structural and functional integrity of GR-LBD. In non-cancer cells the size of GR-LBD is strictly maintained thanks to HSP90's chaperone-guardianship. One should not

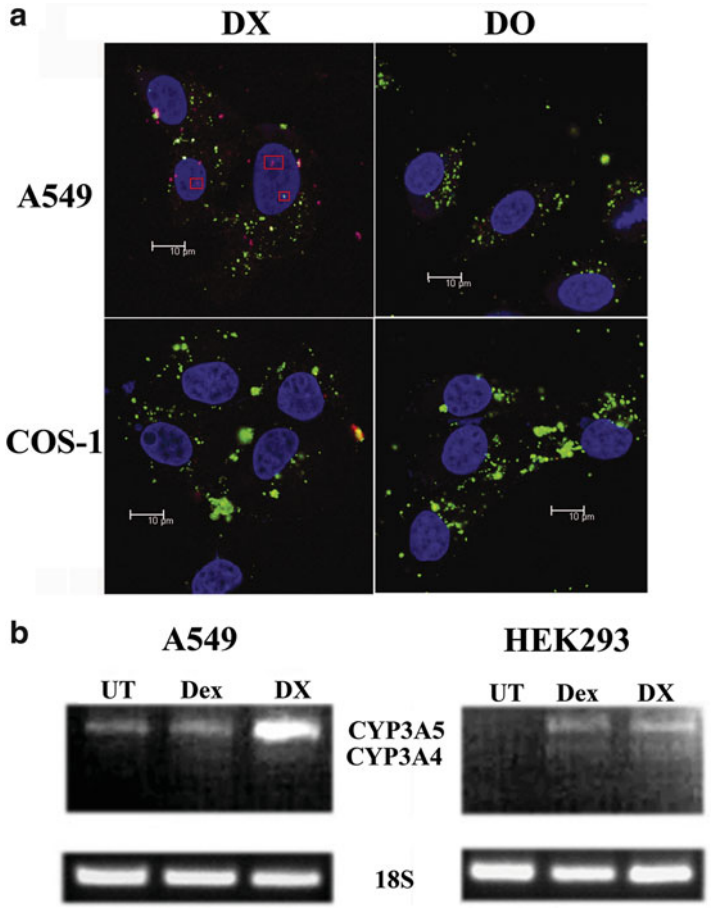



**Fig. 12.2** DX-lipoplex mediated nuclear localization of its lipid component and regulation of GRE genes in cancer cells: A microscopic and reverse transcriptase (RT)-PCR studies. (**a**) A549 (*top row*, cancer cells) and COS-1 (*bottom row*, non-cancer cells) were treated with DX (*left column*) and DO (*right column*) lipoplex containing green-fluorescent lipid and red-fluorescent DNA. Cells were visualized by confocal scanning microscope to ascertain the presence of lipid and/or DNA inside the nucleus. Cells were also visualized following DAPI-based nuclear staining. *White* bar represents 10 μm. The *red* square depicts the localization of lipoplex inside the nucleus. These data shows that DX lipoplex could gain entry in the nucleus of only cancer cells. (**b**) Human cells, A549 (cancer) and HEK293 (non-cancer), were respectively treated with DX and Dexamethasone (Dex) or kept untreated (UT). The total RNAs of the cells were isolated and the m-RNA levels of a GRE-regulated gene, CYP3A5, and another gene, CYP3A4, were determined using RT-PCR technique. Dex has the ability to regulate the expression of only CYP3A5 isoform between these two, 3A5/3A4 isoforms. 18S RNA was used as internal loading control. The respective cDNAs obtained from RT-PCR were run in an agarose gel. These data shows that DX-lipoplex (carrying Dex) could induce cancer cell-specific promotion of many fold more CYP3A5 m-RNA expression than equivalent amount of free Dex (The figure panels were reproduced from [10] with permission from publisher)

forget that even a fraction of DX lipoplex contains multiple numbers of cationic lipids, cholesterol, Dex and DNA. The lipoplex fraction is at least few hundred folds, if not thousand folds bigger than the size of GR-LBD. Additionally, we observed that the DX lipoplex-mediated GR-responsive gene transcription in cancer cells was fivefold to eightfold more than what naked Dex could do (Fig. 12.2b). However, no significant difference in gene transcriptions was observed following DX or Dex-treatment in non-cancer cells.

After observing this DX-mediated, nuclear localization of lipid components followed by excessive GR-transactivation in cancer cells, one can postulate that essentially the chaperone activity of HSP90 in cancer cells is somewhat compromised and understandably, not diminished. Hence, hypothetically this compromised HSP90 allowed the audacious entry of intact DX lipoplex in cancer cells' GR-LBD. This led to unnaturally excessive GR transactivation in cancer cells [10]. To prove, if compromised activity of HSP90 in cancer cells is behind the selective nuclear localization of lipid components of DX lipoplex, we briefly treated non-cancer cells with HSP90 inhibitor geldanamycin and then treated the cells with DX. The lipid components could be then clearly located inside nucleus within few hours. Taken together, we logically hypothesized that since HSP90 in cancer cells cannot be in inhibited state (as it happens by geldanamycin treatment), its chaperone activity must be eternally compromised in cancer cells thereby allowing GR to recognize much larger sized DX lipoplex as a ligand.

Buoyed by the described cancer cell-selective gene transfection we further delivered anticancer p53 gene-coded plasmid to lung tumor subcutaneous model in mice to obtain selective tumor uptake of lipoplex and regression of tumor size [10]. Thus the Dex-associated cationic lipid formulation (DX) paved the development of a novel GR-targeted, cancer-selective, gene delivery agent.

## 12.11 Logic to Target Hsp90: A Cancer Selective Way

No doubt, the cancer cell-selective phenomenon as described above was primarily the handiwork of cellular HSP90 in cancer cells, wherein according to our hypothesis, its chaperone activity is suspected to be compromised. On the basis of this selective, suspected chaperone-activity of HSP90 in cancer cells, we logically questioned if HSP90 can be selectively targeted and say, degraded in cancer cells. Since, HSP90 as a chaperone is linked to many cancer implicated kinases downstream, its selective down-regulation should negatively impact the growth of cancer. If so, how is it possible to target and degrade HSP90 selectively in cancer cells?

As referred above, we have in hand a potent GR-targeted delivery system, which showed highly efficient cancer cell-selective gene transfection. We thought of designing a novel strategy by way of delivering a cargo aimed to adversely affect the expression or activity of HSP90. If HSP90's compromised chaperone-activity in cancer cells is true, then the same activity can be manipulated to affect HSP90's expression or activity. Towards this, one can target HSP90 by many ways

as delineated below. But caution should be maintained about the fact that the strategy should not jeopardize the GR-targeting ability of the liposome, as GR-targeting is the primary goal.

## 12.12 Targeting Hsp90: Possible Ways

### *12.12.1 Small Molecule*

(a) **Hsp90 Inhibitor**
Among the small molecules, HSP90 inhibitors geldanamycin, radicicol and their analogues are well known candidates. The structure and their utility towards targeting HSP90 have already been discussed in an earlier section.

(b) **Trans-Repression of Heat Shock Element (HSE)**
This is a relatively new strategy, wherein molecules [or possibly co-repressor proteins (if any) may be delivered to cells of cancer lineage selectively to act as transcriptional repressor to repress the heat shock elements (HSE) belonging to promoter region of HSPs. The regulation of HSE impacts the turnover of HSP. HSE is hence a lucrative target for regulating the expression and effect of HSP90, especially for treating cancer.

Heat shock factors (HSF) are well known transcriptional regulators for HSEs for the production of HSP. Hence, inhibiting HSF or its activation is certainly a useful anticancer strategy. For the activation of HSF-1, the most important factor yet deciphered in HSE regulation, many other factors are involved. Many proximal negative regulators of HSF activation are well known. These include HDAC6, GSK3α, ERK-1, HSBP-1, etc. and HSP themselves [76–80]. As the list contains HSP, an indiscriminate up-regulation of negative regulators of HSF-1 may have contradicting effects on anticancer result. Moreover, long term and ill-targeted delivery of HSF-1 inhibitor is detrimental for health since HSF-1 protects against neurodegenerative disorders, ischemia etc. [81, 82]. Briefly, new strategies are developed which essentially use different molecular classes of HSF inhibitors that repress HSP activation. They include flavonoids (such as quercetin), quercetin-based prodrugs, benzylidene lactams, diterpene triexpoxide (triptolide), emetine derivatives etc. Some of their mechanisms of action are not clearly known, some act by inhibiting kinases that phosphorylates HSF-1, some act by interfering with transactivation of HSF-1 through acetylation, some sensitizes cancer cells following HSP inhibition, etc. [83–87]. However, for small molecule caution should be exercised for their non-specific delivery to non-cancer cells, including that in brain.

In relation to our newly developed liposomal formulation DX and the present strategy of targeting HSF-1, one thing should be noted. Non-cancer cells also take up GR-targeted formulation (DX) with equal efficiency compared to that exhibited by cancer cells. Moreover, in non-cancer cells the integrity of DX formulation is suspected to be ruptured as a result Dex is freed from lipoplex. This lipoplex-unbound Dex then behaves like free Dex and transactivates GR with same efficiency,

as a free Dex does in cytoplasm [10]. Hence, any small molecule cargo (such as, HSF-1 inhibitors) associated with this formulation would expectedly be freed in non-cancer cells and it might play havoc with the activity of HSP90 leading to collateral damage in normal tissues, unless these molecules are known to exhibit least cytotoxicity in normal cells.

As regard to regulation of HSF-1, GR plays a relatively lesser known, yet a vital role. A GR and HSF-1 crosstalk is discovered which reveals two important things, (a) with assistance from HSF-1, under stress GR's transactivation dramatically increases; (b) in presence of Dex, GR inhibits the cellular heat shock response by inhibiting HSF-1 binding to HSE [88–91]. Notably our previous observation showed that DX formulation has the ability to transcribe GRE-genes multiple folds higher than naked Dex treatment in cancer cells. Moreover, later in the chapter we will discuss that the level of HSP90 in cancer cells comes down upon DX treatment, indicating that DX might influence the expression of HSE-regulated genes (such as HSP90) besides GRE-regulated genes. Although subject to further proof, but going by the previous observation one can reason that with all probability DX will have similar, if not more, inhibitory effect than free Dex on HSF-1-binding to HSE in cancer cells. When DX will additionally carry other anti-HSP90 agent (such as an anti-HSP90 genetic cargo, as is described later) it should doubly impact the expression of HSP90 and an anticancer synergism may be expected.

### *12.12.2 SiRNA/ShRNA*

siRNA or shRNA technology is used to target m-RNA of target protein. Hence, in order to target m-RNA of HSP90 one can design siRNA or shRNA complimenting and targeting m-RNA regions bearing domains of HSP90 preferably conserved among all the isoforms. SiRNAs are synthetic RNAs with perfect complimentary strands to target m-RNA. siRNA usually mediates transient degradation of m-RNA and sometimes causes degradation of off-target m-RNA bearing partial complementarity. This targeting and onset of degradation machinery to eradicate m-RNA occurs in cytoplasm. Hence, one needs an efficient cytoplasmic delivery system, for example cationic liposomes etc. However, cancer cell specific delivery has to be assured to eliminate any possibility of diminishing HSP90 in non-cancer cells. For this, delivery systems targeted to various receptors preferably over-expressed in cancer cells may be employed.

### *12.12.3 Targeting Cells via Over-Expressing Membrane Receptors*

Many cancer cells overexpress receptors, but for efficient targeting and delivery of cargo, delivery systems are logically designed to target over-expressing membrane receptors. Folate receptor, sigma receptor, EGFR, VEGFR, integrin receptors etc.

are over-expressed on cell surfaces of cancer cells belonging to various phenotypes and on the surface of tumor-associated endothelial cells. So, toward delivering small molecules or protein factors selectively to cancer cells one can design delivery systems ornamented on their outer surface with protruding small molecule ligands or antibodies against these respective receptors. Liposomal delivery systems with small molecule ligands targeted to folate receptor, sigma receptor, integrin receptor etc. are well known or recently developed (some representative references including reviews are available, e.g., [64, 92–94]). Respective antibody or corresponding growth factor/protein-ligand linked liposomal delivery systems targeted to EGFR, VEGFR, transferrin receptor are also known (representative examples are [95–97]).

### *12.12.4 Targeting Cytoplasmic Receptors: A Logical Challenge*

Delivery of cargoes exhibiting its cellular fate and action in cytoplasm logically does not need delivery systems targeted to intracellular, cytoplasmic receptors. However, if a delivery is, say, related to targeting estrogen receptors (ER), which is highly implicated in many of the breast cancer incidences, then the challenge assumes an interesting dimension. ER-ligands, natural or synthetic are known to receive help from membrane bound ER-like receptors such as GPR30 for their efficient uptake in breast cancer cells expressing ER. In that case the cellular delivery will be via GPR30, following which the delivery system in all probability is expected to bind to ER for further cytoplasmic activity. However, the integrity of the delivery system in cytoplasm may not remain uncompromised and the binding of delivery system to ER in intact form may not happen. Few years back we showed that estrogen ligand-associated cationic liposomal delivery system could exhibit ER-mediated gene transfection. As a result using anticancer gene as a cargo the delivery system could induce potent killing of ER-expressing breast cancer cells [65]. The killings in ER-negative breast cancer cells were minimal. Although the reason is not clear but these data indicate that somehow in cancer cells the ER-targeted lipoplex might remain intact for which lipoplex could bind to ER and the whole complex could have been ferried inside the nucleus for efficient reporter (or functional, exogenous) gene expression.

### *12.12.5 ShRNA/miRNA Cloned in Plasmid*

For consistent release of silencing RNA in cytoplasm, the siRNA/ShRNA under the influence of an efficient promoter can be cloned in a plasmid. Cationic lipid-based delivery systems exhibiting efficient plasmid transfection can be conveniently used here to deliver and express exogenous gene constructs as RNA (in the form of shRNA or miRNA). This RNA will target the m-RNA of the target HSP90 protein. The delivery of plasmid will ensure sustained release of artificial RNA for a certain time, after which the efficiency vanishes. But the use of shRNA or

miRNA plasmid, producing this synthetic RNA albeit limited for a specified time, is advantageous in one way than using and delivering naked siRNA or shRNA. It will reduce the concept of frequent dosing. A set of siRNA/shRNA so delivered will target a set of m-RNA for the protein. But a plasmid can produce more copy numbers of the same RNA at a given time, repeatedly over a period, and will have an edge over regulating the protein expression turnover. Moreover, siRNA/shRNA for a therapeutic standpoint are needed in huge quantity (and hence are expensive). Additionally, it is a big concern to keep RNA in intact form from endogenous RNAase before its cellular delivery is completed. Hence, shRNA/miRNA cloned in a plasmid is advantageous in many ways. However, since plasmids are produced from bacterial culture it will contain bacterial CpG motifs, which are known to induce non-specific cytokine responses if dosed for a longer period. Moreover, one should positively get rid of possible bacterial toxin contaminants from plasmids before it is used for therapeutic purposes.

## 12.13 Our Approach

### *12.13.1 miRNA Plasmid: The Design Aspect*

Micro-RNAs (or miRNA) are generally 19–24 nucleotide long regulatory RNA molecules. These are produced in cells of most of the eukaryotes from bigger long precursor RNA molecules using RNAseIII complex containing 'Drosha'. The resulting stem loop structure in cytoplasm undergoes 'Dicer'-mediated processing to generate double stranded dsRNA molecules. One of these strands incorporates with Argonaute-associated protein complex (miRNP) to finally interact with target m-RNA bearing partial complimentary sequence to miRNA. The complex then modulates the transcription/translation and hence regulates the corresponding protein expression from m-RNA [98–102]. In this chapter we will describe about artificial mi-RNAs (amiRNA) designed to express as m-RNA utilizing cellular machinery. It will then target m-RNA (with near complementarity) and affect the expression of target protein.

We selected a 21-nucleotide stretch RNA sequence in the 3′UTR region of HSP90 identical in both mouse and human. The idea is to use, in long run, the same construct in human after validating it in animal model. Moreover, the same construct can be possibly used in both allografts as well as human cancer cell-associated xenografts in animals. As depicted in Fig. 12.3a we designed the necessary amiRNA, which will bind mRNA belonging to the target region. Further, a pre-miRNA with flank and loop regions, originally derived from an effectively expressed and processed miRNA, let-7, was also designed (Fig. 12.3b). Finally, the amiRNA sequence was cloned under CMV promoter in pSilencer plasmid vector, which is widely used for miRNA over-expression. Experiments to validate the proper expression and function of amiRNA were performed. This included qRT-PCR of synthesized amiRNA following vector transfection in HEK293 cells as well

the luciferase-based cellular functional studies to see the effective down-regulation of HSP90 3′UTR-luciferase activity. This proved that the amiR-HSP90 plasmid is now ready to be used for further studies.

### *12.13.2 Complexation with Cationic Lipid*

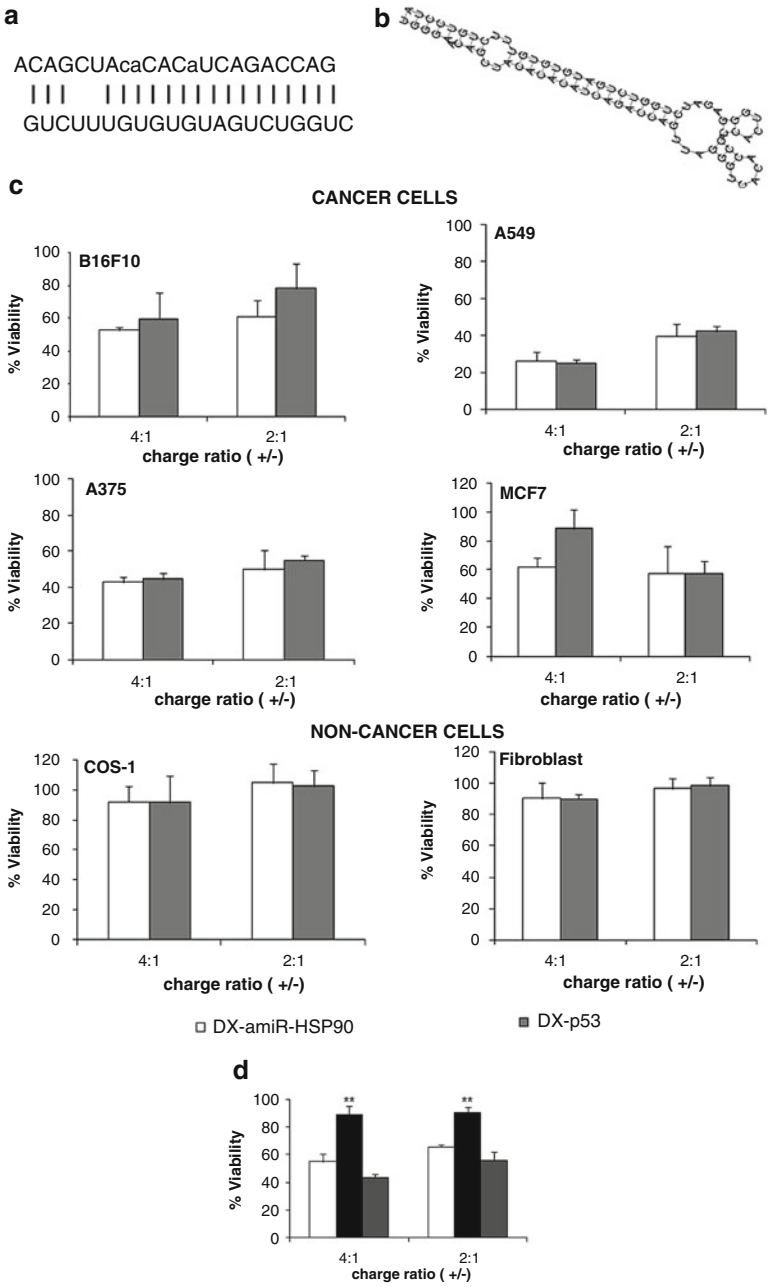
The cationic lipid formulation is the same GR-targeted formulation as we did for our previous work [10] and as described in earlier sections. This formulation DX, contains DODEAC (N,N-Di-n-octadecyl-N,N-dihydroxyethylammonium chloride), cholesterol and dexamethasone at a mole ratio of 1:1:0.75. DX being positively charged was electrostatically complexed with negatively charged amiR-HSP90 pDNA. This resulted in a serum stable, DNAase stable, lipid/DNA complex (or, lipoplex), which maintained a size in the range of 100–200 nm (Supplementary information in [11]). This empirically indicates that the lipoplex would keep DNA cargo intact while maintaining an ideal size for tumor targeting during blood circulation.

### *12.13.3 DX-amiRHSP90 Lipoplex Induces Selective Toxicity in Cancer Cells*

DX-amiR-HSP90 lipoplex was then treated to various cancer and non-cancer cells. As a positive control we used DX-p53 lipoplex and as a negative control we used DX-β galactosidase (DX-β-gal) lipoplex and then we compared the toxic effects of each lipoplexes in various cancer and non-cancer cells. As representatively depicted in Fig. 12.3c, we found the following: (a) DX-amiR-HSP90 lipoplex and DX-p53 lipoplex had similar toxic effects in cancer cells and there were no toxic effects in non-cancer cells; (b) DX-β gal lipoplex had no toxicity in either cancer or non-cancer cells in the given condition (data not shown). The selective toxicity in cancer cells could be because of the selectively high DX-mediated gene transfection in cancer cells. There could be depletion (if any) of HSP90 levels or up-regulated expression of p53 in non-cancer cells, but these may have minimal adverse effect since these cells are perennially under highly regulated surveillance.

### *12.13.4 DX-amiR-HSP90-Induced Toxicity in Cancer Cells is GR-Mediated*

For this to establish, we treated DX-amiR-HSP90 lipoplex to different sets of same cancer cell, first, wherein GR was down-regulated by pretreatment of GR-siRNA and the other where cancer cells were either pretreated with control, scrambled siRNA or kept untreated with any siRNA. The significant reduction in toxicity was


a
ACAGCUAcaCACaUCAGACCAG
GUCUUUGUGUGUAGUCUGGUC
b
c
CANCER CELLS
B16F10
A549
A375
MCF7
NON-CANCER CELLS
COS-1
Fibroblast
% Viability
charge ratio ( +/-)
4:1
2:1
DX-amiR-HSP90
DX-p53
d
**

clearly observed in GR-depleted cancer cells (Fig. 12.3d). The reduction in toxicity was also observed in cancer cells when cells were pretreated with GR-antagonist RU486. No such observations were obtained in non-cancer cells. This clearly indicates that in cancer cells, expressed cytoplasmic GR protein is indeed an essential factor for processing DX-delivered amiR-HSP90 pDNA for their eventual killing.

### *12.13.5 Cancer Cell Selective Regulation of HSP90 Client Protein Kinases*

HSP90 chaperones multiple kinases, which are highly implicated in cancer progression and sustenance. Down-regulation of HSP90 levels in cancer cells will adversely affect the functioning of each or many of these kinases since HSP90 is involved in chaperoning them simultaneously. A transient decrease in HSP90 will have more impact in cancer cells than in normal cells because of these over-working tendencies of HSP90 in cancer cells. So, let us see in cellular level what could happen to different kinase client proteins of HSP90 in cancer cells.

Upon DX-amiR-HSP90 lipoplex treatment there were clear down-regulation of HSP90 in both mRNA and protein levels in cancer cells (Fig. 12.4a). It is to be noted that first of all, it was not easy to show down-regulation of HSP90 proteins in cultured cancer cells. We could show the HSP90 down-regulation in freshly isolated tumor cells obtained from tumors created by those cultured cancer cells only. This indicates that tumor cells in tumor acquire certain properties, which make them more dependent on HSP90. If so, then subtle change in HSP90 level in tumor (or tumor-associated cancer cells) should have distinctly more effect than that expected in normal cultured cells. The data above shows that using DX-amiR-HSP90 lipoplex one can possibly try to dissect the role of HSP90 in tumor microenvironment.

However, the kinases such as AKT and MAPK of both freshly isolated tumor cells and cultured cancer cells showed signs of down-regulation with concomitant increase in cleaved caspase 3 level (Fig. 12.4b). This indicates that the anticancer effect was triggered by down-regulation of pro-proliferative, HSP90 client, kinase

**Fig. 12.3** Design of the artificial micro-RNA (amiRNA) against *Hsp90* 3′UTR and viability studies using this amiRNA: (**a**) predicted binding pattern of the amiR to the *Hsp90* 30UTR, (**b**) predicted secondary structure of the pre-miRNA incorporating flanks and loop region of miR-let-7 and the amiRNA. This is then cloned in a plasmid (namely, amiR-HSP90) and then used to complex with DX liposome, (**c**) viability studies in cancer cells, B16F10, A549, A375 & MCF-7 and in non cancer cells, COS-1 & NIH3T3 fibroblast. Cells were continuously treated with DX-amiR-HSP90 (*white bar*) and DX-p53 (*gray bar*) lipoplexes for 96 h. Following this MTT assay was performed to assess the viability of respective cells. (**d**) Viability of A549 cells initially kept untreated or treated with respective siRNAs followed by 96 h treatment of DX-amiR-HSP90 lipoplex. *White*, *black*, and *gray* bars represent siRNA untreated, GR siRNA treated, and negative control siRNA treated cells, respectively (The figure panels were reproduced from [11] with permission from publisher)

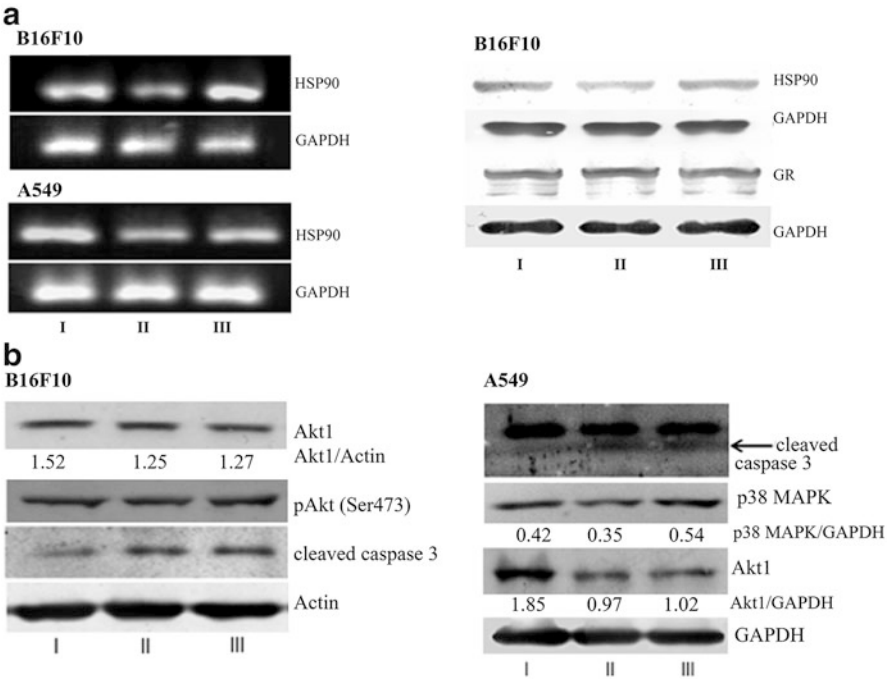



**Fig. 12.4** Expression levels of HSP90, its client proteins and caspases in cancer cells: (**a**) Hsp90 m-RNA levels in both cancer cells, B16F10 and A549 (*left panels*) and Hsp90 and GR protein levels obtained after treatment of cultured tumor cells freshly isolated from the B16F10 tumor developed in mice (*right panel*). (**b**) In here, Western blot analysis of B16F10 and A549 cell lysates is exhibited. Cell lysates were obtained from cells either kept untreated (I), or continuously treated with DX-amiR-Hsp90 (II), and DX-βgal (III) for 36 h (m-RNA levels) or 48 h (protein levels). DX-βgal is a control lipoplex that carries a non-therapeutic control plasmid encoding $\tilde{\beta}$ galactosidase enzyme. This figure clearly shows that with down-regulation of m-RNA for HSP90 and levels for HSP90 proteins obtained from freshly isolated tumor cells, various HSP90 client proteins, especially kinases (Akt1, MAPK) are down-regulated. As a result, apoptosis is triggered in respective cancer cells as witnessed by the up-regulation of cleaved caspase-3 (Reproduced with publisher's permission from [11])

proteins leading to induction of intrinsic mode of apoptosis. In here, two additional things are to be noted: (a) Even if HSP90 down-regulation is unobservable (in cultured cancer cells), the down-regulations of kinase proteins were clearly observed; (b) DX-β-gal lipoplex also induced Akt down-regulation and formation of cleaved caspase-3, even though the anticancer readout was not as prominent as DX-amiR-HSP90 lipoplex treatment. The data so far could not directly explain these phenomena. However, as discussed earlier, Dex critically up-regulates GR-mediated inhibition of HSF-1-induced heat shock response and consequently down-regulates expression of HSPs [88]. We found that in cancer cells DX formulation transactivate GR many folds more than even Dex (Fig. 12.2b and ref. [10]). Hence, in view of the above observation regarding DX-β-gal lipoplex we hypothesize that since this

lipoplex consists of DX it could have induced Dex-alike, if not more, inhibitory effect on HSF-1 and hence on the expression HSP90 (even though it is not clearly observable), thereby resulting in down-regulation of HSP90 client protein Akt.

### *12.13.6 Effect of DX-Lipoplex In Vivo*

After accomplishing DX-amiR-HSP90-mediated down-regulation of HSP90 and its client protein kinases in cancer cells we studied the effect of this lipoplex in tumor. Tumor is an extremely multifarious condition with multiple factors involved to maintain its complexities. Hence, treating tumor is not straightforward compared to killing cancer cells in culture. However, our understanding was that since HSP90 is involved in so many growth-regulating pathways of both tumor-associated epithelial and vascular endothelial cells through chaperoning several of its member proteins, tumor cells may be more dependent on HSP90, as many of these inter-related pathways are simultaneously switched on and may relentlessly cross-talk to make the environment more and more complex. As a result, even a subtle change in HSP90 levels may induce deep-running effects on tumor by affecting the growth of tumor-associated epithelial and vascular endothelial cells.

With this pre-notion we treated DX-amiR-HSP90 to two different models in mice bearing subcutaneous tumor of melanoma and lung cancer cells respectively. Clearly, as depicted in Fig. 12.5a, DX-amiR-HSP90 lipoplex had maximum effect in reducing the tumor growth. The control lipoplex (DX-βgal) also showed some tumor-growth inhibiting effect in both the models, indicating the possible GR-mediated heat shock response inhibiting effect of individual DX formulation entity.

The enormity of tumor size reduction can be seen by the representative images of tumor from individual treatment groups (Fig. 12.5b). The melanoma tumor was a very aggressive model wherein mice in untreated groups die within 30 days. We find that in this experiment even though untreated mice were dead by 25 days, the mice in other treated groups showed no signs of mortality for more than 45 days (data not shown).

Microscopic visualization of sections of tumors from DX-amiR-HSP90-treated mice revealed few interesting features (Fig. 12.5c). Tumor cells underwent apoptosis as revealed by TUNEL assay (green fluorescent areas). Additionally on marking tumor-associated vascular endothelial cells with VE-cadherin (red fluorescent areas) we found that these cells also underwent apoptosis (yellowish regions). To trace the reason for this effect we find that the tumor sections bearing vascular endothelial cells bore fewer vascular endothelial growth factor receptor-2 (VEGFR2) on its surface [11]. VEGFR2 is another client protein of HSP90. It is highly expressed in aggressive tumors, which exhibit features of immense angiogenesis. Evidently, if the HSP90 level was lowered it would eventually affect the level of VEGFR-2 and we think this is what we witnessed. With the lowering levels of VEGFR-2 on vascular cells there would be no uptake of vascular endothelial growth factors (VEGF). As a result the paracrine growth stimulation and vascular permeability in these cells

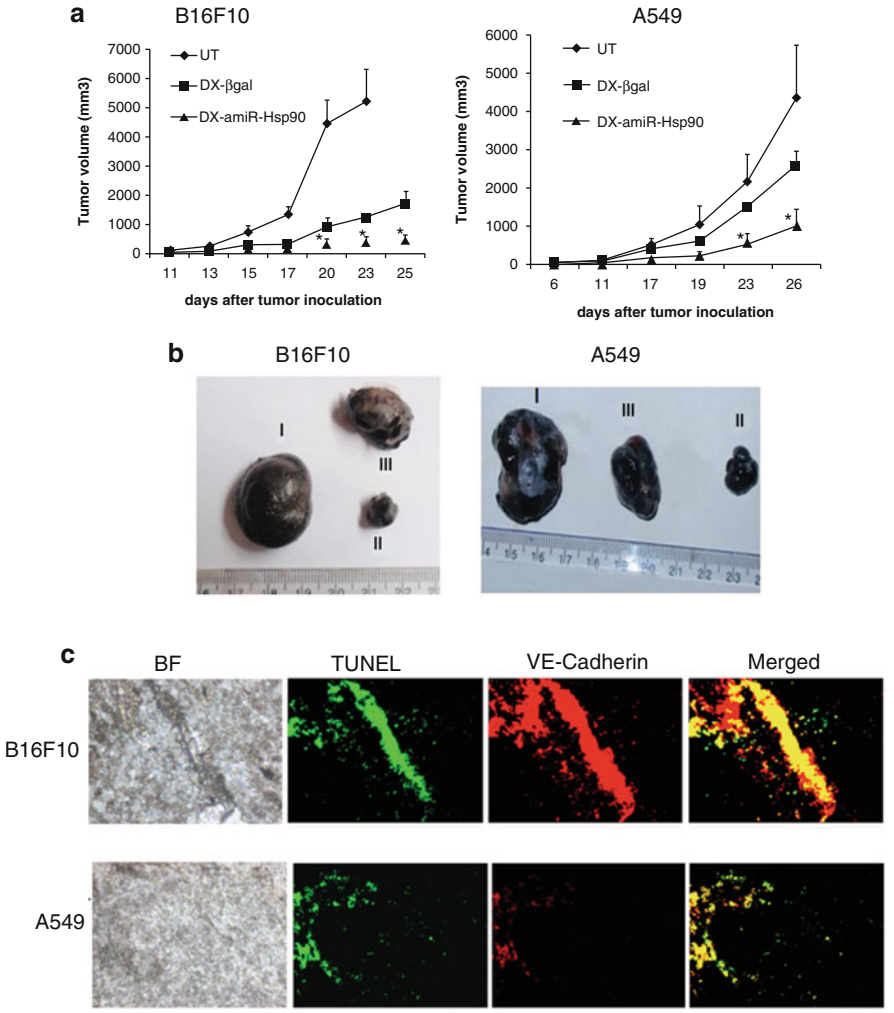



**Fig. 12.5** Tumor growth curve, representative tumor sizes and microscopic images for signatures of apoptosis in sections of tumors: (**a**) Tumor growth curve after subcutaneous implantation of B16F10 (*left panel*) cells in C57BL6/J mice and A549 cells (*right panel*) in BALB/C athymic nude mice followed by intraperitoneal injection of 5 % glucose (UT, *black rhombus*), DX-βgal (*black square*), and DX-amiR-Hsp90 (*black triangle*) lipoplex at 6:1 cationic lipid/DNA charge ratio. Intraperitoneal injections started from day 8 for both the models. Seven injections were given with 2 days interval. Y-axis denotes the size of tumors as tumor volume in cubic mm, and X-axis denotes the number of days passed after tumor inoculation. The *asterisk* (*) denotes $p < 0.01$ while comparing with DX-βgal treatment. (**b**) This exhibits the image pictures of representative samples of B16F10 and A549 tumors excised after sacrificing the mice on day 25 and 26 respectively for models as described in section A. Herein, (I) denotes tumors from untreated, (II) denotes tumors from DX-amiR-Hsp90 lipoplex treated and (III) denotes tumors from DX-βgal lipoplex treated groups respectively. (**c**) Microscopic pictures of 10 mm tumor sections of DX-amiR-Hsp90 treated group from B16F10 and A549 tumor. First column from left shows the tissue architecture in bright field (BF), second column from left shows the apoptotic regions in TUNEL assay

would hinder, thereby reducing the diffusion of vital proteins (needed to support cell's migration) in the interstitial space within tumor. Tumor microenvironment supports the formation of micro-angiogenesis, which needs the help of this paracrine stimulation from vascular endothelial cells. Hence herein, reduction in VEGFR2 level sequestered overall angiogenesis, which led to eventual shrinkage of tumor.

Till now we saw downsizing of tumors in DX-amiR-HSP90 lipoplex treated tumor-bearing mice. But one may logically question if tumor inhibitory effect is really due to the real time expression of anti-miRNA against HSP90 in tumor or not. Clearly, there was no direct evidence to show that DX-lipoplex did have the ability to selectively express the genetic cargo in tumors, relieving other vital organs. For this, we chose to deliver a reporter plasmid using DX. The reporter plasmid contained gene clone of green fluorescent protein (GFP) under the influence of CMV promoter. It is expected that on a reasonable time scale upon gene delivery green fluorescent protein would functionally express in various organs. Therefore, we injected DX-GFP lipoplex in tumor bearing mice and after 24 h we collected the organs including tumors from sacrificed mice. Organs were sectioned and visualized under microscope. We observed practically no GFP expressions in any vital organs including lung, liver, kidney, spleen and heart. But, expression of GFP was abundant in tumors (Fig. 12.6). This proved that DX, which carries GR ligand Dex, has the ability to functionally express its genetic cargo in only tumors thereby nullifying any non-specific genetic expression and therefore any possibility of collateral damages in vital organs. This data can be related to potent expression of anti-miRNA against HSP90 in tumors if delivered by DX in vivo.

Next, we show that DX-amiR-HSP90 lipoplex delivery in vivo led to changes in protein levels of various pro-proliferative and apoptotic factors in tumor mass (Fig. 12.7). Down-regulation of HSP90 was logically evident. Possibly as a result, Protein kinase B or Akt was also down-regulated leading to down-regulation of its different phosphorylated forms. Expression of one of the vital HSP90 client proteins, Bcl-2 was inversely affected, as a result the cascade of events related to triggering of intrinsic pathway of cancer, such as elevation of cleaved form of caspase 9 and intact caspase 3 were observed. Cleaved caspase 3 was not evident though, but we observed another interesting feature. The expression of apoptosis-inducing p53 protein, a protein prone to undergo ubiquitination and degradation in tumor, was the highest in DX-amiR-HSP90 group.

p53 is a client protein of HSP90, so do Akt. Upon HSP90 down-regulation Akt was down-regulated but p53 was up-regulated. This contradiction although links to

**Fig. 12.5** (continued) (*green fluorescent*), third column from left shows the regions of where endothelial cells are present and are visualized after staining with VE-cadherin antibody (*red fluorescent*), and extreme right column is merged pictures of second & third columns from left. All the images are taken at 10x objective magnification. This merged section shows that upon the treatment of DX-amiR-HSP90 signs of apoptosis could be detected clearly in tumor cells (*green areas*) as well as tumor-associated endothelial cells (*yellow areas*). The emergence of yellow area additionally indicates the anti-angiogenic role of DX-amiR-HSP90 lipoplex (Selective image and figure panels are reproduced from [11] with permission)

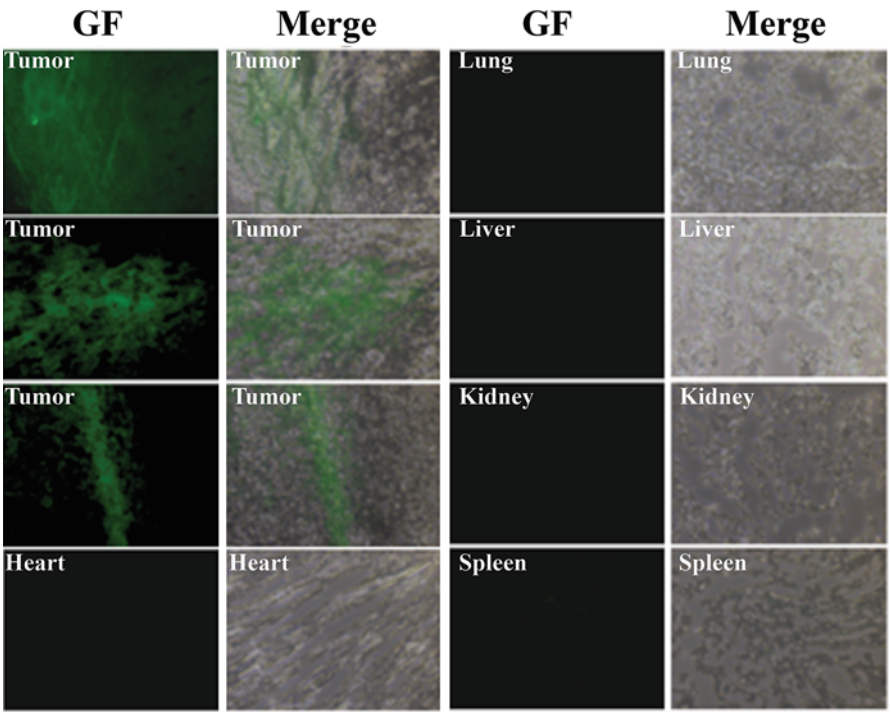



**Fig. 12.6** Functional bio-distribution of DX-lipoplex in tumor-bearing mice: Sections (10 μ thickness) of tumors and different vital organs were obtained after sacrificing B16F10 tumor-bearing mice intraperitoneally injected with DX-associated green fluorescence protein (GFP)-plasmid. The sections from various organs and tumors of mice treated with lipoplex (DX-GFP) were mutually compared. The respective imaging of sections from individual organs and tumors from DX-GFP treated mice were obtained at same exposure time and magnification (10×). 'GF' represents green field and 'Merge' represents merging of respective sections visualized under bright field and under GF (Selective image panels were reproduced from [11] with permission). This data clearly shows that DX-lipoplex has a very selective ability to induce gene expression of its genetic cargo in tumor-associated cells. DX-lipoplex has apparently no capability to transfect normal cells of vital organs (Selective image panels are reproduced from [11] with permission)

eventual antitumor effect but could not be explained by the present data. Moreover, there are contradicting reports about the status of p53 in the tumor cells of interest here. B16F10, the melanoma cell, carries wild type p53 [103–106]. This p53 status is however also contradicted but it seems p53 maintains its function and retains its binding activity to the response elements in these melanoma cells [107]. Our data however indicates that p53 in this experimental set of B16F10 cells is possibly wild type, otherwise the up-regulation could have resulted in tumor aggression rather than tumor retardation. Others have also found that geldanamycin-induced inhibition of HSP90 leads to down-regulation of mutant p53 but up-regulation of wild type p53 [108, 109]. One can further speculate several other things: Firstly, Akt phosphorylates mdm2, which eventually ubiquitinates p53 for degradation.

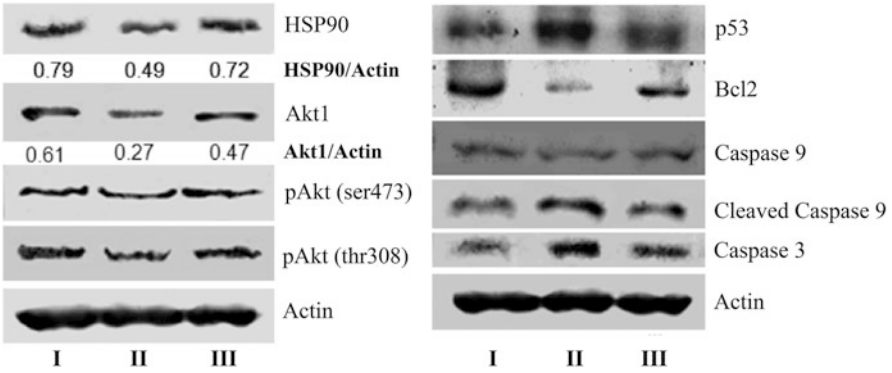



**Fig. 12.7** Western blot analysis of tumor lystates: Differential expression of different regulators of apoptosis and proliferation in B16F10 tumor lysates from the tumors of untreated group (I), from groups treated with DX-amiR-HSP90 (II), and DX-βgal (III) respectively. As a whole, the figure clearly depicts that upon HSP90 down-regulation, levels of various regulators of apoptosis (Bcl-2, caspases) and that of protein kinase (Akt1) are changed. Pro-cancerous factors such as Bcl-2 and Akt-1 are down-regulated, at the same time signatures of apoptosis (i.e., the caspases) are up-regulated (Reproduced *in toto* from [11] with permission)

Here, as Akt was down-regulated, up-regulation of p53 was expected. It could be possible that up-regulation of p53 possibly out-weighed p53's down-regulation (if any) by HSP90 depletion. The second reason could be the following: There are two main isoforms of HSP90. HSP90α is a stress-induced one whereas HSP90β is constitutively expressed isoform and both their elimination is bad for any cell's survival. However, it is also known that ratio of α/β isoform changes predominantly in favor of α under stress condition, such as during malignant transformation [110]. The client proteins, such as survival signaling kinases (Akt), oncogenes (v-src, Bcr-Abl), whose up-regulated presence provides stability and perpetuity to this malignant transformation are mostly chaperoned by HSP90α. Interestingly, p53 is chaperoned by HSP90β [46]. Our construct was designed to target a common region of 3′UTR of mRNA of both α and β isoform. Although subject to realization but stoichiometric analysis should show larger proportions of α isoform than β isoform in the tumor. As a result, following amiR-HSP90 treatment, expectedly more of α isoform would be eliminated than β isoform and hence more of α-isoform-chaperoned client proteins would be down-regulated.

## 12.14 Any Other Possible Target for Gene Therapy and Other Approaches

In this subsection, we would like to emphasize some hypothetical strategies that may be adopted for targeting HSP90. The first one is, targeting the highly conserved middle domain of HSP90. It contains the catalytic Arg380 motif, which interacts

with the ATP γ̃phosphate trapped inside the closed, catalytically active N-terminal dimerized state. Hydrolysis of ATP is the key function for chaperone activity of Hsp90. Arg380 while residing in a highly conserved domain helps in this hydrolysis. The domain covering Arg380 is hence a lucrative region for gene therapy and can be targeted by suitably designed shRNA or miRNA.

Secondly, it is known that GR-LBD is chaperoned by HSP90. The release of this client protein (i.e., GR) from HSP90 requires ATP hydrolysis. In turn, the GR-LBD affects the nucleotide-binding affinity of HSP90. It increases affinity for ATP and decreases for ADP. Another client protein is p53. It remains in bound state with HSP90. Interestingly, p53-HSP90β dissociation requires binding of ATP but not its hydrolysis. Chaperoning of client proteins such as kinases requires ATP binding. So, GR could be a hypothetically potential target whose critical down-regulation can affect nucleotide binding to HSP90 and may have indirect effect on client protein kinases. However, as far as cancer treatment is concerned there are possible contradicting data against this theory of down-regulating GR. It is shown that Dex-treatment induces energetically unfavorable gluconeogenesis over glycolysis in cancer cells thereby rendering these cells drug sensitive [111]. This shows that presence of GR can be utilized for cancer treatment. Our recent discovery along this line is worth mentioning. We found that the formulation DX has the capability to induce many folds more GR-transactivation (which involves gluconeogenesis also) than even Dex (Fig. 12.2b). Hence, we hypothesize that DX (or any cationic lipid formulation with a GR ligand) will have more drug-sensitivity-inducing effect than Dex in cancer cells. This possibility is also worth testing.

Thirdly, our recent experience, as described above, allows us to recommend using this artificial miRNA strategy to specifically target conserved 3′UTR domains of HSP90α isoforms only. As discussed above, α-isoform is critically up-regulated in tumors and this isoform is critically involved in chaperoning various cancer implicated kinases, whereas the β-isoform is involved in chaperoning p53, the apoptosis-inducing gene. So, targeting α-isoform over β-isoform may have better implications toward treating cancer. However, it is also to be noted that, going by the HSP90 addiction theory, the dependence of mutated p-53 on β-isoform is more than its wild type form. Hence, depending on p53 status in cancer cells the strategy of utilizing certain design of miRNA may be conveniently adopted to obtain optimal anticancer outcome.

## 12.15 Conclusion

In this chapter, we described the development of a novel concoction of DX-amiR-HSP90 formulation that exhibited the first gene therapy against HSP90 in a cancer selective manner. The artificial miRNA against HSP90 directly inflicted down-regulation of HSP90 expression in only cancer cells. Notably, the strategy does not involve inhibition of HSP90 activity rather involved direct down-regulation of HSP90 protein expression. We know cancer cells are HSP90 ‘addicted’. As a result

of even slightest down-regulation of HSP90 expression, the pro-proliferative client proteins of HSP90 in cancer cells including various kinases showed down-regulated expression, which was possibly enough to reduce the tumor burden. This selective reduction of tumor burden was possible when we delivered the genetic cargo via GR. The use of GR or its antagonists for cancer treatment are limited. Our data suggests that GR can be selectively manipulated for cancer treatment. It also indicates that the GR-manipulation may have additional effect on HSP90 down-regulation possibly via inhibition of heat shock factors. Even GR-targeting molecules such as Dex if formulated with cationic lipid showed HSP90 down-regulatory effect, indicating that GR should come under the active scanner for targeting HSP90 for developing newer anticancer modalities.

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