Evaluation of prostate-specific antigen as a novel biomarker of Hsp90 inhibition

Katerina Oikonomopoulou a,b,c, Antoninus Soosaipillai a, Eleftherios P. Diamandis a,b,c,*

a Samuel Lunenfeld Research Institute and Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, Toronto, Ontario, Canada M5T 3L9
b Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada M5G 1L5
c Department of Clinical Biochemistry, University Health Network and Toronto Medical Laboratories, Toronto, Ontario, Canada M5G 2C4

Received 15 May 2009; received in revised form 2 July 2009; accepted 7 July 2009
Available online 23 July 2009

Abstract

Objectives: Hsp90 inhibition has already been evaluated by assessing the expression of proteins, like the androgen receptor (AR) and the IGFBP-2. We hypothesized that AR-regulated proteins, such as prostate-specific antigen (PSA), may serve as biomarkers for Hsp90 inhibition.

Design and methods: We utilized the androgen-stimulated BT-474 breast cancer cells to trigger PSA secretion, which was quantified by ELISA. PSA concentration was used to evaluate the potency of an experimental compound (NVP-AUY922), in comparison to the commercially available 17-allylamine-17-demethoxygeldanamycin (17-AAG) Hsp90 inhibitor.

Results: PSA concentration was reduced in a dose-dependent manner (2-fold more than IGFBP-2) and was accompanied by AR decrease. Utilizing PSA expression as a marker for Hsp90 inhibition, we concluded that the novel NVP-AUY922 inhibitor was about 8-fold more potent than 17-AAG.

Conclusions: This study showed that PSA may serve as a sensitive biomarker of Hsp90 inhibition and may aid in selecting new chemotherapeutics. Furthermore, the novel Hsp90 inhibitor was highly potent, suggesting that it may be an attractive agent for clinical trials.

© 2009 The Canadian Society of Clinical Chemists. Published by Elsevier Inc. All rights reserved.

Keywords: Androgen receptor; Heat shock protein 90; Hsp90 inhibitors; Human kallikrein-related peptidase; Prostate-specific antigen

Introduction

Heat-shock protein 90 (Hsp90) is an abundantly expressed molecular chaperone involved in maintaining conformational stability, maturation, and activity of its numerous interacting partners known as clients [1,2]. Hsp90 chaperone couples with selected co-chaperone proteins to form dynamic multi-protein complexes, which can bind ATP. The client proteins are chaperoned within the Hsp90 complex. Inhibitors of Hsp90 have the ability to affect client stability by competing for the binding site of ATP and regulating Hsp90 interactions with its co-chaperones and client proteins [1,3]. This ultimately results in the decreased stability/activity of the client proteins, which become susceptible to degradation via proteasome-dependent pathways.

Hsp90 client proteins are mainly involved in signal transduction, cell cycle control and transcriptional regulation [3]. These processes are critical for the function of normal and cancer cells. For instance, Hsp90 clients can play significant roles in all essential alterations, which lead to the transformation of a normal cell into cancerous, including self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of apoptosis, unlimited replicative potential, sustained angiogen-
esis, tissue invasion, and metastasis [3,4]. Indications exist to show that Hsp90 formation of its mature structure and increased chaperone activity may depend on the malignant potential of a cell [5]. Its active conformation is especially important for the tumour cells to ensure maximum expression and/or activity of clients. Therefore, Hsp90 has emerged as an attractive therapeutic target, because it represents a pleiotropic single molecular target that has the capability of simultaneously affecting the six major hallmarks of cancer cells [6].

Geldanamycin and its analog 17-allylamino-17-demethoxygeldanamycin (17-AAG) belong to a group of Hsp90 inhibitors known as the benzoquinone ansamycins [7–9]. These compounds inhibit Hsp90 function via their competitive binding to the ATP binding site of Hsp90, which results in reduced ATPase activity and degradation of the Hsp90-associated client proteins. This class of inhibitors has a higher affinity for “active” Hsp90 chaperone complexes, which are present in cancer cells, resulting in potent anti-tumour activity [9–11]. Further investigation of the therapeutic potential indicated that the hepatotoxicity and the narrow therapeutic window of geldanamycin precluded its clinical development [12]. However, the reduced hepatotoxicity of 17-AAG has led to clinical studies with some promising results, although various limitations exist for its clinical applicability, such as its low oral bioavailability, cumbersome formulation and variable metabolism by polymorphic enzymes [13]. Other unrelated molecules that target the ATPase activity of Hsp90 are either less potent than 17-AAG or exhibit unfavourable pharmacological properties and in vivo instability [14]. The quest, therefore, has been on the discovery of new, more potent Hsp90 inhibitors, which can fully take advantage of the pleiotropic potential of Hsp90 as a therapeutic target in vivo with minimal adverse side effects.

Several studies have stressed the importance of identifying biological markers to evaluate potential Hsp90 inhibitors before they can enter clinical trials [15–18]. In principle, the same markers may be valuable in assessing the level of Hsp90 inhibition clinically and assisting in the management of cancer patients according to their therapeutic response. Thus far, Hsp90 inhibition has mainly been monitored by following the expression/integrity of Hsp90 clients by western blot. Nevertheless, these studies have focused primarily on intracellular proteins as potential markers of Hsp90 inhibition. Recently, Zhang et al. proposed two extracellular proteins, namely the insulin-like growth factor-binding protein 2 (IGFBP-2) and the human epidermal growth factor receptor 2 extracellular domain (HER-2 ECD), as potential clients regulated by Hsp90 inhibition [18].

Taking into consideration the advantage of utilizing a secreted protein as a marker of Hsp90 inhibition, similarly to Zhang et al. [18], we hypothesized that expression of secreted prostate-specific antigen (PSA/KLK3), an established cancer biomarker, can be regulated by Hsp90 inhibitors. We assumed that this effect could be due to instability of the androgen receptor, which is a known Hsp90 client [9,19], and a critical regulator of PSA expression in the presence of androgens [20]. We further hypothesized that PSA quantification by a highly sensitive and specific ELISA methodology could be used as a sensitive readout to assess the potency of novel synthetic Hsp90 inhibitors.

Materials and methods

Cell lines

The breast cancer cell line, HTB-20 (BT-474) [21], and the prostate cancer cell line, CRL-1740 (LNCaP) [22], were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). Cell lines were cultured in RPMI 1640 medium (Gibco BRL, Gaithersburg, MD, USA), supplemented with fetal bovine serum (10%), in 6-well plates. Cultures were maintained at 37 °C in a humidified 5% CO₂ atmosphere until the cell monolayers became confluent. At approximately 80% confluency, cells were washed and medium was replaced with CDCHO serum-free medium (Gibco), supplemented with 8 mM t-glutamine (Invitrogen Canada Inc., Burlington, ON, Canada). Cells were hormonally stimulated or not and incubated with or without various concentrations of Hsp90 inhibitors (details are described below) for 24–48 h. Stimulation and inhibitor treatments were performed in three cell replicates derived from at least three or more independently grown cultures. After incubation, supernatants were collected and stored at −20 °C until use.

Reagents

For stimulation experiments, dihydroxytestosterone (DHT; Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) or Norgestrel (Sigma) dissolved in 100% ethanol was added to the culture medium, at a final concentration of 0.1 μM. Cells stimulated with ethanol were included as controls. The final ethanol concentration was less than 1% in all cases. The Hsp90 inhibitor, 17-allylamine-17-demethoxygeldanamycin (17-AAG), was purchased from Sigma (catalogue number A8476). Another Hsp90 inhibitor, AUY922 (NVP-AUY922; [23]), was kindly provided by Novartis (Novartis Institutes for BioMedical Research, Cambridge, MA, USA). Compounds were diluted in anhydrous DMSO (Sigma).

Optimization of cell culture and inhibitor treatment

The optimal cell density, concentration and duration of inhibitor treatment, as well as the optimal concentration of hormonal stimulation were established using the following five assays: (a) estimation of absolute cell counts using a fluormocytometer, (b) a 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay to determine mitochondrial activity as an indication of cell viability (Sigma, catalogue number TOX-1), (c) a lactate dehydrogenase (LDH) assay to measure the increased LDH content of cell supernatants as a result of cell death (Sigma, catalogue number TOX-7), (d) a Coomassie total protein assay as an estimate of cell number (Thermo Scientific, Rockford, IL, USA) and a control for internal variation, and (e) a commercially available enzyme-linked immunosorbent assay (ELISA) for quantification of
insulin-like growth factor-binding protein 2 (IGFBP-2) (R&D Systems, Minneapolis, MN, USA; catalogue number DY674), a suggested biomarker of Hsp90 inhibition [18].

Monitoring Hsp90 inhibition at optimal conditions

Cell supernatants were analyzed with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions (NuPage 4–12% Bis–Tris gels; Invitrogen). Proteins were transferred to a Hybond-C Extra nitrocellulose membrane (Amersham Biosciences, Pittsburgh, PA, USA) and blocked with 5% milk and 0.1% Tween-20 in Tris-buffered saline (TBST) overnight, at 4 °C. Subsequently, they were incubated with either an antiserum against the androgen receptor (AR; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA, catalogue number sc-816) or a monoclonal antibody against β-actin (Santa Cruz, catalogue number sc-47778) for 1 h in 1% milk-TBST. All primary antibodies were diluted 2,000-fold. Membranes were then washed with TBST, and treated with the respective goat secondary ALP-conjugated antibody (Jackson ImmunoResearch, West Grove, PA, USA; 1:5,000 dilution). Blots were finally visualized with Immulite Chemiluminescent substrate treatment (Diagnostic Products Corp, Los Angeles, CA, USA) for 10 min.

In addition, IGFBP-2 expression was examined, as described above, as a marker of successful blockade of Hsp90-related pathways at optimal cell culture and treatment conditions. The protein levels of vascular endothelial growth factor (VEGF; R&D Systems, catalogue number QVE00B) and Cathepsin D (Calbiochem-EMD Biosciences, Inc., San Diego, CA, USA, catalogue number QIA29) were also quantified to determine their potential as biomarkers of Hsp90 inhibition. Further, the expression of prostate-specific antigen (PSA) was assayed by utilizing an in-house specific ELISA, as previously described [24]. In brief, recombinant PSA protein calibrators, diluted in bovine serum albumin (BSA) buffer (6% BSA, 50 mM Tris, and 0.05% sodium azide, pH 7.8), and samples were added in white polystyrene 96-well plates, pre-coated overnight (room temperature) with 100 μL of coating antibody solution (50 mM Tris buffer, 0.05% sodium azide, pH 7.8, containing 250 ng/well of anti-PSA monoclonal antibody). Where appropriate, sample dilutions were also performed in 6% BSA buffer. Assay buffer [50 μL of 50 mM Tris, 6% BSA, 0.01% goat IgG (Sigma), 0.005% mouse IgG (Fortron Bio Science Inc, Morrisville, NC, USA), 0.1% bovine IgG (Sigma), 0.5 M KCl, and 0.05% sodium azide, pH 7.8] was further added to each well and incubated for 2 h with shaking at room temperature. The plates were washed six times with the washing buffer (5 mM Tris buffer, 150 mM NaCl, and 0.05% Tween-20, pH 7.8) and 100 μL of biotinylated detection antibody solution (25 ng/well of anti-PSA monoclonal antibody in assay buffer) were added to each well and incubated for 1 h at room temperature with shaking. The plates were then washed six times with the washing buffer. Subsequently, 5 ng/well of streptavidin–alkaline phosphatase (SA-ALP) solution (Jackson ImmunoResearch) in BSA buffer were added to each well and incubated for 15 min at room temperature. The plate was washed six times with washing buffer and the substrate (100 μL of 0.1 M Tris buffer, pH 9.1, containing 1 mM diflunisal phosphate, 0.1 M NaCl and 1 mM MgCl2) was added to each well and incubated for 10 min at room temperature. Finally, developing solution (100 μL of 1 M Tris, 0.4 M NaOH, 2 mM TbCl3, and 3 mM EDTA) was added for 1 min at room temperature. The Tb3+-fluorescence was measured on a time-resolved fluorometer, the Cyberflur 615 Immunoanalyizer (MDS Nordion, Kanata, ON, Canada). Calibration and data reduction were performed automatically. This PSA assay has a detection limit of 1 ng/L and coefficients of variation <10% within the measurement range of 1–10,000 ng/L [24].

Results

Optimal growth conditions of BT474

The main aim of this study was to identify sensitive markers of Hsp90 inhibition in cancer cells. We utilized a breast cancer cell line, the proteome of which has been previously delineated by our group [25]. LNCaP was also used, since the mechanism of AR stabilization, due to the Hsp90 chaperone function, and the consequent secretion of PSA has been thoroughly characterized for this cell line [26,27]. The optimal growth conditions of the BT-474 cells were determined as described under “Materials and methods”. Under basal conditions (no steroid hormone stimulation) the BT-474 cell line produced and secreted minute amounts of PSA (<5 ng/L; data not shown). Hormonal treatment of BT-474 cells with DHT (an androgen) or Norgestrel (an androgenic progestin) for 48 h triggered an increase of prostate-specific antigen concentration in the supernatant of these cells, as determined by ELISA (>100 ng/L) (Figs. 1A and B, [17-AAG]=0).

PSA versus other candidate proteins as biomarkers of Hsp90 inhibition

To assess the level of inhibition and construct the dose-response curves for 17-AAG, we measured IGFBP-2 as previously described [18]. Taking into account our formerly established list of proteins expressed by BT-474 cells [25], we selected two other secreted proteins for evaluation, namely the vascular endothelial growth factor (VEGF) and Cathepsin D. We found that the levels of both IGFBP-2 and VEGF were decreased in BT-474 cell supernatants after 17-AAG treatment in a dose-dependent manner (Figs. 1C and D), while concentration of Cathepsin D levels remained unaffected (data not shown). The level of IGFBP-2 inhibition was independent of hormonal stimulation and similar to the one reported by Zhang et al. [18].

We also measured prostate-specific antigen (PSA) with our in-house developed ELISA assay. Optimal results were obtained with parallel treatment with androgen and 17-AAG in serum-free media; these conditions resulted in a steep dose-response curve (Figs. 1A and B). The half maximal inhibitory concentration (IC50) of 17-AAG calculated from the PSA expression data was lower than the one calculated from the IGFBP-2 data; for example at a dose of 0.01 μM 17-AAG, PSA...
expression was reduced by 40%, whereas IGFBP-2 expression decreased by only 17% (Fig. 1E). Similar analysis of 17AAG-treated LNCaP cells revealed about 25% inhibition of PSA secretion which was inferior to the one observed in BT-474 cells (data not shown).

**Hsp90 inhibitors target PSA secretion via the AR pathway**

We hypothesized that treatment of BT-474 cells with 17-AAG (resulting in Hsp90 inhibition) can cause inactivation or proteolysis of the androgen receptor, leading to a remarkable

---

Fig. 1. Dose-response curve of PSA decrease after Hsp90 inhibition. BT-474 cells were grown to 80% confluency and treated with 17-AAG in serum-free media in the presence (A–C; filled symbols) or absence (C, D; open symbols) of hormone for 24 (without hormonal co-stimulation) or 48 (with hormonal co-stimulation) h. Stimulation was performed with 0.1 μM of either Norgestrel (A, C, E) or DHT (B) and indicated protein content of cell supernatants was quantified as described in Materials and methods. In panel E, PSA and IGFBP-2 expression is presented as % from baseline (no 17-AAG) for comparison purposes. The data are representative of three or more independently conducted experiments for each condition. For more explanations, see the text.
decrease in the expression of PSA upon androgen stimulation. To investigate this hypothesis, lysates of BT-474 cells treated with 17-AAG plus androgen were immunoblotted with an androgen receptor polyclonal antibody. AR immunoreactivity of cell lysates and secreted PSA concentration were markedly reduced at concentrations of 17-AAG greater than 0.1 μM (Figs. 2A and B). Expression of the control protein, β-actin, was not significantly altered. This data indicates that the decrease of secreted PSA upon 17-AAG treatment is due to PSA transcriptional downregulation, as a result of AR decrease.

**PSA as a marker of efficacy of Hsp90 inhibitors**

Using secretion of PSA as an index of Hsp90 inhibition, the efficacy of a novel experimental inhibitor of Hsp90 (NVP-AUY922 or AUY922) was assessed, in comparison to the commercially available 17-AAG. The dose-response curves of 17-AAG and AUY922 are shown in Fig. 3. LDH was also quantified in BT-474 supernatants to establish the concentrations of Hsp90 inhibitors that were non-toxic for the cells (Fig. 3). The potency of the novel Hsp90 inhibitor (AUY922) to reduce PSA expression at inhibitor concentrations lower than 0.005 μM (non-toxic at these levels) was greater than the potency of 17-AAG, with AUY922 exhibiting an IC₅₀ of about 0.001 μM.

**Discussion**

To investigate whether PSA production can be considered as a suitable biomarker of Hsp90 inhibition, we used the BT-474 (breast) and the LNCaP (prostate) cancer cell lines. Both cell lines harbour a functional androgen receptor (AR). LNCaP produces large amounts of PSA in the absence of AR stimulation, since the AR has a critical point mutation that facilitates nuclear localization independently of androgen stimulation [27]. It has been suggested that the endogenously high basal levels of PSA in LNCaP cells may result from the Hsp90-mediated stabilization of the mutant AR, since treatment with 17-AAG results in reduced PSA expression [27]. However, the mechanism of AR activation in the absence of ligand remains unclear. As well, geldanamycin and its analog 17-AAG have been shown to inhibit androgen-stimulated PSA expression in LNCaP cells [26,27].

BT-474 is a breast cancer cell line originating from a solid, invasive ductal breast carcinoma [21], which is positive for both estrogen and progesterone receptors [20,28]. It also possesses functional androgen receptors [28], which is reflective of more than 50% of breast cancer cases [29]. This cell line also maintains an amplification of HER-2/neu or erbB-2 [30], which represents 30% of all breast cancer cases [31]. The BT-474 cells secrete significant amounts of PSA after hormonal stimulation (Figs. 1A and B, [17-AAG]=0), in agreement with our previous findings [28], and representative of 30% of breast tumours [32]. The assay was developed using the BT-474 cell line, since proteomic analysis indicated that these cells abundantly express both AR and Hsp90 [25].

Given these observations, we considered that PSA expression may be AR-Hsp90-dependent in the BT-474 cell line, similar to the LNCaP. The ELISA utilized has low detection limit (≤1 ng/L) and equimolarity for the free PSA and the PSA-antichymotrypsin complex [24]. These properties allow for sensitive detection of minimal changes in total PSA concentration of cell supernatants. Despite the variability of the response of our cells to hormonal stimulations conducted with independently grown cells, we clearly demonstrated that PSA decrease after 17-AAG treatment (Figs. 1A and B) was superior to the decrease observed with IGFBP-2 (Fig. 1C), which is a recently proposed biomarker of Hsp90 inhibition in BT-474 cells [18]. Presumably, after binding of the hormone, the activated hormone-AR complex undergoes a conformational change, dissociates from the chaperone complex and translocates to the nucleus, where it regulates the expression of AR-responsive genes, such as PSA. Inhibition of Hsp90 may result in the release of AR and its potential degradation by the proteasome, rendering the cells refractory to hormonal stimulation, thus reducing or abolishing PSA production (Fig. 2). Our speculative model, which takes into account previous reports describing the regulation of AR by Hsp90 inhibitors [26,27], is shown in Fig. 4. In support of our data, one of these studies found reduced PSA mRNA levels in prostate cancer cell lines co-treated with AR activators and 17-AAG, due to degradation of the AR [27]. However, the possibility of additional non-AR mechanisms that may regulate PSA secretion in BT-474 cells cannot be fully excluded.

Current assays for Hsp90 inhibitors are based on the competitive binding of compounds to recombinant Hsp90 protein.
preparations [33]. Our assay has two unique characteristics over competitive binding assays: (a) the readout (reduction of PSA concentration in cell supernatants) monitors not only the ability of an inhibitor to bind Hsp90, but also to facilitate the degradation of a well-characterized client (the androgen receptor) that regulates PSA secretion; for this reason, this method is likely to identify a greater number of lead compounds in high-throughput settings, since it is not limited to inhibitors of the ATPase activity of Hsp90 alone, and (b) the background signal, especially for cells that do not endogenously produce PSA (such as the BT-474 breast cancer cells) is extremely low, suggesting that the assay can generate steep dose-response curves, and thus, afford high sensitivity. Other advantages include the reproducibility, precision and convenience of our PSA ELISA [24,34], as well as its low cost and straightforward adaptability to high-throughput settings.

The emergence of Hsp90 as a promising new therapeutic target has led to the discovery of several inhibitory compounds with potent anti-tumour activity. Unfortunately, these compounds have, thus far, had limited success in clinical trials, restricted by the toxicity of the drugs and their formulations [13,15–17,35], or they have shown inconsistent efficiency for accessing Hsp90 inhibition [36]. Regardless, Hsp90 remains an exciting and attractive target for cancer therapy, because it represents a single molecular target capable of simultaneously regulating multiple cancer-associated proteins, the functions of which promote the acquisition of the necessary alterations required for carcinogenesis [3–6].

One of our objectives was to rank the potency of the experimental Hsp90 inhibitor by using the expression of PSA as an index of functional androgen receptor (Fig. 3). 17-AAG had an IC50 of more than 8 nM, while the IC50 of the novel inhibitor AUY922 was about 1–5 nM. Thus, this assay could aid in the development of improved chemotherapeutics. The toxicity of AUY922 to the cells was similar to 17-AAG (Fig. 3). Further, our inhibitor was utilized at concentrations 5-fold lower than previously reported compounds [35,37]. It is interesting to note that AUY922 has been recently shown to possess significant anti-proliferative activity against cancer cells and in tumour xenografts, with an IC50 of about 3–5 nM in the case of BT-474 [23,38,39].

A variety of studies have indicated the potential of Hsp90 inhibitors for treatment of many cancer types. The basic therapeutic strategy is based on the use of multiple drugs concurrently, making it more difficult for tumours to develop resistance to the selected drug regimen. For instance, tumour cells that develop resistance to imatinib mesylate (Gleevec™, Novartis Pharmaceuticals) remain sensitive to the Hsp90 inhibitors geldanamycin and 17-AAG [40,41]. Such molecules are attractive in compensating for the propensity of...
tumour cells to develop drug resistance to conventional and/or targeted agents. Furthermore, Hsp90 inhibitors have been used in combination with several DNA damaging agents (such as etoposide and topoisomerase II inhibitors) resulting in an enhanced radio-sensitization (and DNA damage-induced apoptosis) of cancer cell lines derived from a variety of histological cancer types [10,11]. The development of a sensitive assay to aid in the discovery of novel inhibitors of Hsp90 activity for cancer suppression or treatment is, therefore, a high priority. In the future, this assay could be applied to other breast cancer-derived cell lines (e.g. MDA-MB-468 cells) or cell lines derived from other types of tumours in order to monitor Hsp90 inhibition using additional secreted markers expressed by these cells (e.g. KLK5 and other kallikrein-related peptidases).

Acknowledgments

We are grateful to Novartis Institutes for BioMedical Research (Cambridge, MA, USA) for providing the Hsp90 inhibitor NVP-AUY922 (AU922), as well as to Dr. Alan Buckler and Dr. Chantale T. Guy, at Novartis, for their valuable input.

References


Fig. 4. Schematic representation of Hsp90-inhibition, degradation of the AR and subsequent decrease of PSA secretion. Hsp90 is coupled to several co-chaperone proteins and ATP to form multi-protein complexes. Client proteins, such as the androgen receptor (AR), are chaperoned within the Hsp90 complex. Under hormonal stimulation (e.g. with DHT or Norgestrel), the androgen receptor is released and translocates to the nucleus to facilitate expression of genes, such as PSA. This results in increased concentration of the secreted PSA protein. Inhibition of the Hsp90 complex by Hsp90 inhibitors, such as 17-AAG, results in the early release of AR, which renders the receptor vulnerable to degradation by the proteasome. Under these conditions, the cells remain non-responsive to hormonal stimulation and PSA expression is minimal. ATP is indicated, since the model inhibitor 17-AAG inhibits Hsp90 by competitive binding to its ATP binding site. IP, 23, Hsp70 (70) and “other” represent co-chaperones of Hsp90, while other co-factors were excluded for simplicity. The model was adapted from Zhang et al. [3].


