Rational Design and Synthesis of Androgen Receptor-Targeted Nonsteroidal Anti-Androgen Ligands for the Tumor-Specific Delivery of a Doxorubicin−Formaldehyde Conjugate

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The synthesis and preliminary evaluation of a doxorubicin−formaldehyde conjugate tethered to the nonsteroidal antiandrogen, cyanonilutamide (RU 56279), for the treatment of prostate cancer are reported. The relative ability of the targeting group to bind to the human androgen receptor was studied as a function of tether. The tether served to attach the antiandrogen to the doxorubicin−formaldehyde conjugate via an N-Mannich base of a salicylamide derivative. The salicylamide was selected to serve as a trigger release mechanism to separate the doxorubicin−formaldehyde conjugate from the targeting group after it has bound to the androgen receptor. The remaining part of the tether consisted of a linear group that spanned from the 5-position of the salicylamide to the 3′-position of cyanonilutamide. The structures explored for the linear region of the tether were derivatives of di(ethylene glycol), tri(ethylene glycol), N,N′-disubstituted-piperazine, and 2-butyne-1,4-diol. Relative binding affinity of the tethers bound to the targeting group for human androgen receptor were measured using a ^{3}H-Mibolerone competition assay and varied from 18% of nilutamide binding for the butynediol-based linear region to less than 1% for one of the piperazine derivatives. The complete targeted drug with the butynediol-based linear region has a relative binding affinity of 10%. This relative binding affinity is encouraging in light of the cocrystal structure of human androgen receptor ligand binding domain bound to the steroid Metribolone which predicts very limited space for a tether connecting the antiandrogen on the inside to the cytotoxin on the outside.

Introduction

Although doxorubicin has been used extensively in the clinic over the past 3 decades, its use is still limited by severe acute and chronic systemic toxicities.[1] Indeed, the primary failure of the age of cytotoxic chemotherapy has been the unacceptable side effects observed with the agents employed.[2] Extensive efforts in the field of developmental therapeutics have focused on new paradigms of chemotherapy for selective toxicity to developing tumors with minimal systemic side effects.

The strategies investigated to achieve the desired tumor specificity are many and varied yet, to date, the vast majority have been greatly unsuccessful. Immunotherapy is perhaps the most promising of these approaches on account of the natural complexity and perpetual evolution of the immune system, as well as its inherent ability to recognize aberrant cell growth. Unfortunately, the power of the immune system has yet to be successfully harnessed for the conquest of cancer in humans.[3]

Other methods being explored include the inhibition of developing intratumoral vasculature (tumor angiogenesis) and the direct interference with tumor cell metabolism via nontoxic ligands for receptors expressed in cancerous cells. The concept of targeting tumor angiogenesis was initially proposed by Folkman three decades ago;[4] yet, only in recent years has it received vast attention in the literature. The initial attraction to antiangiogenic drugs was the lack of resistance mechanisms incurred by normal endothelial cells of the tumor vasculature upon exposure to chemotherapeutic agents. Unlike tumor cells, which can adjust to and overcome a cytotoxic assault, the cells of the requisite tumor vasculature are not prone to mutation and, therefore, ultimately succumb to chemotherapy.[5] Unfortunately, the tumors themselves have found ways to develop artificial vasculature and grow in the absence of authentic blood vessels.[6] Despite such set backs, antiangiogenic molecules are still of considerable interest in the field and offer great promise for the future; however, drugs of widespread clinical use have yet to be identified.[5]

While there has been limited success in the development of nontoxic, protein-specific molecules, such as Gleevec (Novartis, Switzerland), which selectively kill cancerous cells, these agents are generally active against only certain types of neoplasms.[7] The majority of tumors, which do not depend on the targeted proteins for growth, do not succumb to these new drugs. Although the development of nontoxic antitumor drugs is still in its infancy and holds great promise for the design of future therapeutics, there is an inherent shortcoming in the mode of action of these drugs. The genetic instability of cancerous cells leads to widespread mutations in cellular proteins involved at all levels of metabolism and growth.[8] The selectivity required of

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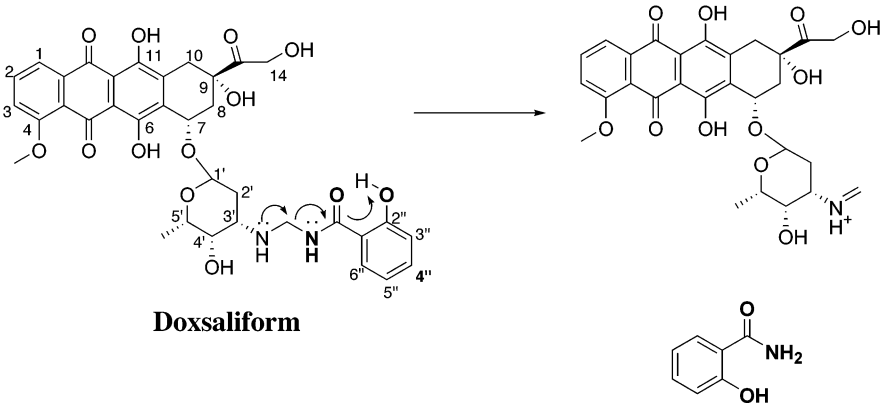
Scheme 1. Release of the Formaldehyde Schiff Base of Doxorubicin from Doxsaliform



Doxsaliform

these tumor-targeted drugs is dependent on binding to a single, specific receptor expressed by malignant cells. Mutation or overexpression of the targeted receptor or circumvention of its participation in metabolic pathways will ultimately lead to drug resistance. Indeed, this phenomenon is already coming to light in the clinic.[9]

Still another approach to achieving antitumor specificity with concomitant reduction of systemic toxicity is the selective delivery of cytotoxins. While the targeting of cytotoxic agents to tumors via a carrier molecule is relatively new to the clinic, much preclinical work has been carried out in this promising field. Cytotoxins as varied as nitrogen mustards,[10] nitrosoureas,[11,12] anthracyclines,[13−15] taxanes,[16,17] mitomycin C,[18] membrane acting peptides,[19] and assorted antibiotics[20,21] have all been employed in the search for tumor-selective therapeutics. Although these selective cytotoxins rely upon the expression of specific protein targets and are, therefore, prone to resistance mechanisms such as mutation or changes in expression of the target, they have several advantages over related nontoxic ligands. While the efficacy of molecules which interfere with the action of a specific cellular protein depend on expression of the target in every cell of a tumor, targeting compounds which release a nonspecific cytotoxin can potentially act upon tissue surrounding the target-expressing cell. Accumulation of the cytotoxin within the tumor is the goal, as opposed to direct action of the ligand on a cellular receptor. Ligands which act to merely deliver a cytotoxin may even be expected to exploit established resistance mechanisms such as overexpression of the targeted receptor.[9,22]

Work in our laboratory, and others, has shown that the concomitant delivery of formaldehyde with doxorubicin, or other anthracyclines, to growing tumor cells leads to a superior antiprolific response relative to the delivery of doxorubicin alone.[23−25] In an attempt to capitalize on this observation, we have recently developed several unique prodrugs of a formaldehyde conjugate of the anthracycline doxorubicin. We propose that the partial hydrolysis of these prodrugs renders the doxorubicin−formaldehyde Schiff base, which subsequently serves to covalently modify genomic DNA, an event proposed to ultimately be more toxic than the mere intercalation of unmodified doxorubicin. From among these novel prodrugs we have identified a candidate for development as a tumor targeted source of both doxorubicin and formaldehyde. The N-Mannich base resulting from the condensation of doxorubicin with salicylamide (2-hydroxybenzamide) and formaldehyde, nominally referred to as doxsaliform (Scheme 1), has proven to be a superior cytotoxin, relative to the parent drug, against both doxorubicin-sensitive and -resistant cultured human tumor cells.[26] The N-Mannich base construct has been observed to be inactive when intact, but upon time-dependent partial hydrolysis, yields the superactive doxorubicin−formaldehyde Schiff base. This partial hydrolysis occurs with a half-life of 57 min under physiological conditions, yet can be readily increased via acyloxymethylation of the salicylamide phenolic moiety. It is the salicylamide fragment of this N-Mannich base which we have here modified via the attachment of a tumor-targeting moiety.

The androgen receptor (AR) has been identified in a wide array of human tumors in both male and female patients. Carcinomas of the breast,[27,28] ovary,[29] esophagus,[30] lung,[31] and prostate[32] have all been shown to express the androgen receptor. The AR exists primarily as a cytosolic receptor[33] in complex with several heat-shock proteins (hsp70, hsp90, and hsp56−59). Ligand binding leads to dissociation of the heat-shock proteins, homodimerization, and translocation into the nucleus where the dimeric receptor recognizes hormone-responsive elements and various components of the transcription machinery.[34,35] The receptor is often overexpressed in hormone refractory prostate cancer and is also known to acquire mutations which lead to promiscuous binding of various nonandrogen ligands.[22,34] Our intention was to exploit these characteristics of the androgen receptor via the synthesis of a series of nonsteroidal antiandrogens which may be used to deliver a doxorubicin−formaldehyde conjugate to AR-expressing tumors.

Results and Discussion

Chemistry. Doxorubicin has had only limited success in the treatment of prostate cancer in the clinic.[36] However, several groups have shown that the targeting of DOX to prostate-derived neoplasms via prostate-specific antigen (PSA) leads to not only preferential accumulation of the drug in these tissues, but also leads to a profound response, relative to untargeted drug, as indicated by serum PSA levels and tumor mass in treated mice.[37,38] While individual PSA enzymes are expected to activate several of the targeted prodrugs, and thus allow for a continuous targeting effect, the enzyme is extracellular and releases the targeting group from the cytotoxin outside of the cell. We hope to utilize the mechanism of AR nuclear translocation as a means

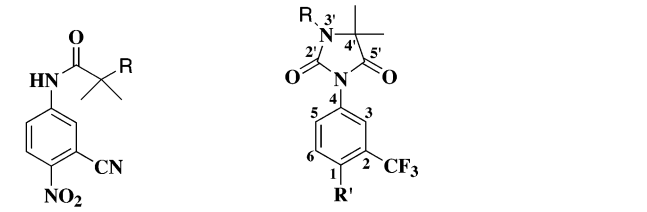


Figure 1. Various nonsteroidal antiandrogens derived from flutamide (**1a**) or nilutamide (**2a**).

of not only localizing the prodrug in tumors but also as a method of delivering a superactive doxorubicin−formaldehyde conjugate to the nuclei of expressing cells, thereby necessitating a smaller dose of the cytotoxin.

A variety of both steroidal and nonsteroidal ligands for the AR have been described, providing many potential options to exploit as AR targeting molecules.[33,39] Our hope was to develop a targeting platform based on endogenous ligands or known therapeutic agents so as to take advantage of preestablished pharmacokinetics, pharmacodynamics, toxicology, and metabolic mechanisms. The obvious choice for an AR-specific ligand may at first appear to be dihydrotestosterone (DHT). While the endogenous steroid is enticing, it is characterized by many drawbacks. The hydrophobic nature of steroid drugs does not facilitate aqueous dissolution; conjugation of DHT to doxorubicin is not expected to yield a molecule of acceptable solubility. Also of concern is the fact that DHT is, by definition, an AR agonist. The use of a growth-promoting agent to deliver a cytotoxin is not expected to be an efficient method of inhibiting tumor growth. Most disconcerting about the use of derivatized DHT, or other steroids, as targeting groups are the poor binding affinities of these molecules for the AR. Several groups have synthesized various derivatives of DHT, generally modified at the 17 position or by etherification or esterification of the 17-hydroxy group.[40−42] With few exceptions, steroidal analogues have been shown to exhibit less than 10% the AR binding affinity of DHT.

The nonsteroidal antiandrogens (NSAs) bear little resemblance to the endogenous steroids they antagonize. Most notably, they are smaller and are characterized by functional groups which lead to a considerably more polarizable surface area relative to the steroidal ligands. Although the clinically employed NSAs exhibit decreased AR binding affinity relative to DHT, binding can be readily improved through facile modifications of the core structures.[43−45] Because of these aspects, as well as the general ease of synthesis, we chose to explore the modification of NSAs through the introduction of varying tethers for the attachment of the salicylamide trigger.

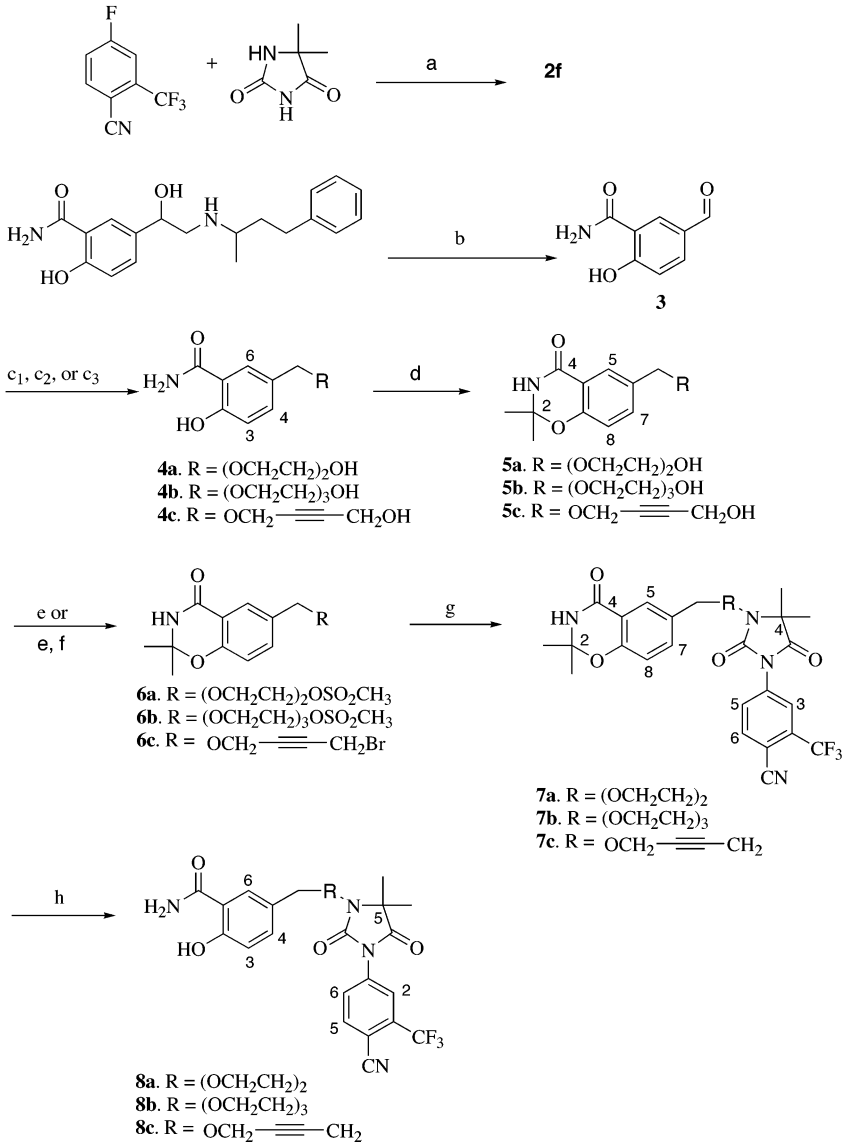
Nilutamide **2a** (Figure 1) is one of a small group of clinically employed antiandrogens. Discovered in 1979, nilutamide is classified as a pure anti-androgen.[46] Unlike the most commonly employed clinical antiandrogen, flutamide **1a**, which acts as a partial agonist and actually promotes growth of AR-expressing cells at higher concentrations,[33] nilutamide shows no growth enhancing characteristics.[47] Of considerable interest is the observation that the 3′ nitrogen of the 1-cyano derivative of nilutamide can be modified with a wide variety of substituents (Figure 1) which lead to improved binding over the parent drug. The binding pocket of the AR apparently not only tolerates, but positively interacts with substituents such as primary alcohols of varying lengths, double and triple bonds, and aromatic ring systems.[44,45,48] While the direct attachment of doxorubicin to nilutamide may not yield a viable ligand for the androgen receptor, the accommodating nature of the AR ligand binding domain is expected to allow for the development of a suitable tether by which nilutamide may be linked to salicylamide. A construct of this type not only allows for the concomitant delivery of doxorubicin and formaldehyde via preparation of an N-Mannich base with the tethered salicylamide, but also renders a generic targeting group which may be used to deliver a variety of other compounds to AR-expressing cells.

Prompted by the superior AR binding affinity of the alcohol **2d** (Figure 1), relative to nilutamide and hydroxyflutamide, the active metabolite of flutamide,[45,48] our initial efforts were aimed at the synthesis of a series of ethylene glycol-derived tethers. Poly(ethylene glycol)s are commonly used excipients for drug delivery. They are well tolerated and relatively stable to metabolic enzymes.[49] Tethers consisting of di(ethylene glycol) and tri(ethylene glycol) were explored based on their varying lengths and steric similarities to the hydroxybutane arm of **2d**. The straight chain ethers were expected to occupy the same cleft of the androgen receptor ligand binding domain (AR-LBD) in which the hydroxybutyl chain of **2d** resides. The ethylene glycols were also expected to offer superior aqueous solubility relative to simple homologous alkyl tethers. The ethylene glycol dimer and trimer were both employed in an effort to identify a tether of sufficient length to preclude interference of ligand binding by the salicylamide and anthracycline portions of the final drug.

The introduction of functional groups to salicylamide to allow for tether attachment is no trivial task. Candidate derivatives must have an electronic character similar to the parent unsubstituted salicylamide. The presence of electron-donating or -withdrawing groups which are in conjugation with the aromatic salicylamide core threatens to alter the known time constant for partial hydrolysis of doxsaliform. Although a longer or shorter time frame for release of the doxorubicin−formaldehyde Schiff base may ultimately yield a superior therapeutic agent, the current series of targeted drugs was designed for direct comparison to the predetermined cytotoxicity of the untargeted doxsaliform. Also of concern was the metabolic stability of the tether. Attachment of the tethers to salicylamide via a benzylic ether was ultimately explored in an attempt to address these issues and yielded a stable construct.

Synthesis of the targeting group with di(ethylene glycol) and tri(ethylene glycol) tethers was conducted as shown in Scheme 2. The 1-cyano derivative of nilutamide **2f** was prepared in one step and 60% yield from 4-fluoro-2-(trifluoromethyl)benzonitrile and 5,5-

Scheme 2[a]

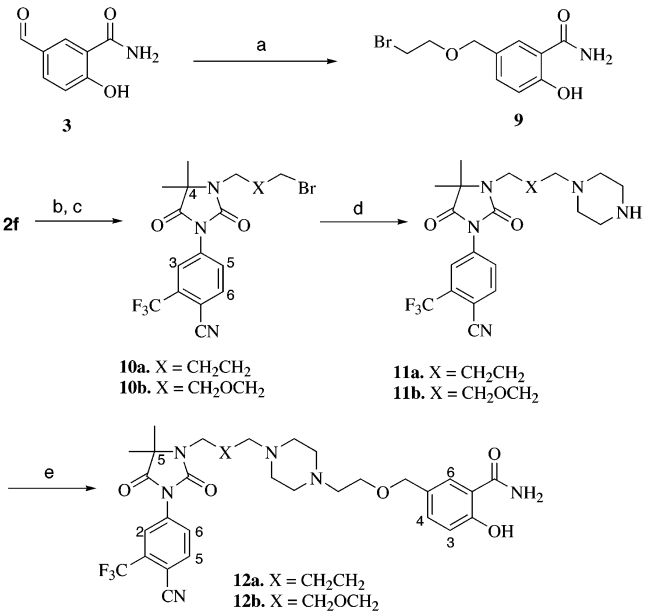


[a] (a) K_2CO_3, DMF, 65 °C; (b) $NaIO_4$, $NaHCO_3/H_2O$ pH 8.0; (c_1) $B_{10}H_{14}$, di(ethylene glycol), (c_2) $B_{10}H_{14}$, tri(ethylene glycol), (c_3) $B_{10}H_{14}$, 2-butyne-1,4-diol; (d) 2,2-dimethoxypropane, p-TsOH, acetone; (e) MsCl, TEA, THF; f) LiBr (10 equiv); (g) sodium salt of **2f**, 55 °C; (h) p-TsOH, $MeOH/H_2O$, reflux.

dimethylhydantoin in the presence of potassium carbonate. The oxidation of Labetalol with sodium periodate was accomplished using a modified literature procedure to give 5-formylsalicylamide **3** in 70% yield.[50] Introduction of the tethers, to generate the alcohols **4a** and **4b**, was then carried out in good yields (up to 91%) via decaborane mediated reductive etherification using the respective ethylene glycol as solvent.[51] Protection of the amide and phenolic moieties of **4a** and **4b** to give the dimethylbenzoxazines **5a** and **5b** was achieved in up to 88% yield by reflux in acetone and 2,2-dimethoxypropane, containing a catalytic amount of *p*-toluenesulfonic acid.[52] The primary alcohol of each of the benzoxazine-protected intermediates was then mesylated in 88−92% yield by treatment with triethylamine in the presence of pyridinum methanesulfonate, formed in situ, to give compounds **6a** and **6b**. Coupling of **2f** and tether bearing salycilamide portions of the targeting group was accomplished by deprotonation of **2f** with sodium hydride followed by addition of either **6a** or **6b.** The resulting benzoxazine-protected targeting groups **7a** and **7b** were then deprotected by reflux in methanol containing 20% water, in the presence of a catalytic amount of *p*-toluenesulfonic acid, to yield the desired compounds **8a** and **8b**.

Despite the solubilizing effect of the ethylene glycol tethers, we feared that the presence of three hydrophobic aromatic ring systems in the final prodrug, namely doxorubicin, salicylamide, and the NSA **2f**, would lead to problems with the formulation of this series of compounds. There were two major concerns with the ethylene glycol tethers. First was the possibility that the inherent insolubility of the intact drugs would prohibit dissolution in aqueous media. Of equal concern was the scenario in which the drug would display some degree of solubility but would fold upon itself to exploit favorable π-stacking interactions between **2f** and either the doxorubicin or salicylamide portions of the prodrug.[53] A second set of constructs was therefore devised in order to introduce a solubilizing functionality and a source of rigidity into the tether. The heterocyclic diamine piperazine was chosen in an effort to address

Scheme 3[a]



[a] (a) $B_{10}H_{14}$, 2-bromoethanol; (b) NaH, DMF; (c) 1,4-dibromobutane or bis(2-bromoethyl) ether, 60 °C; (d) piperazine, THF, reflux; (e) **9**, TEA, THF, reflux.

both concerns. The introduction of two ionizable amines into the tether should afford additional solubility relative to the uncharged ethylene glycols. Also, the conformational constraints imposed by the six-membered piperazine ring should serve to inhibit intramolecular associations of the drug. The syntheses of two derivatives incorporating piperazine into the tether are presented in Scheme 3. Deprotonation of **2f** with sodium hydride in DMF followed by addition of an excess of either 1,4-dibromobutane or bis(2-bromoethyl) ether yielded the brominated compounds **10a** and **10b** in 85% and 82% yields, respectively. Subsequent displacement of the bromide leaving group with excess piperazine in tetrahydrofuran gave the diamino derivatives **11a** and **11b**. Finally, the target compounds **12a** and **12b** were prepared by refluxing **11a** or **11b** in THF with the 2-bromoethoxy ether **9**, which was prepared by the same route as the ethylene glycol-derived benzylic ethers.

Although the four described compounds, **8a**, **8b**, **12a**, and **12b**, were expected to be sufficient to allow for preliminary evaluation of our targeting strategy, a final candidate was pursued in an attempt to capitalize on noted attributes of previously characterized AR binding molecules. Danishefsky et al. have recently described a series of testosterone−geldanamycin conjugates which show a wide range of efficacy, dependent solely upon the length of an alkynyl tether employed to join the two drugs.[20] Danishefsky demonstrated that a β-propargylic group at the 17-position of testosterone is necessary for biological activity in the tested series. Presumably, the triple bond serves to stringently direct the tether's protrusion from the binding pocket. The relevance of this requirement for tether rigidity in testosterone−geldanamycin conjugates to NSA derivative binding was not immediately clear. There is no direct evidence to suggest that the tethers of Danishefsky's conjugates reside in the same cleft in the AR binding pocket as do the 3′ substituents of the series **2b**−**e**. However, much indirect evidence supports this very assertion.

Although little structural information about the AR was available when the work described here was initiated, crystallographic data on the ligand binding conformation of the AR were independently reported by Miller et al.[39] and Carrondo et al.[54] while the syntheses of our targeting groups were being conducted. While the crystallographic data further indicated that an appropriate tether could be accommodated by the AR, it also suggested that the receptor would not tolerate constructs of considerable steric bulk. Using homology modeling techniques employing the X-ray crystal structure of the highly homologous progesterone receptor (PR) LBD, Miller et al. proposed a 3-dimensional conformation for nilutamide bound to the AR-LBD.[39] In this model, the 3′ nitrogen of nilutamide is shown to occupy the same space in the binding pocket of PR as does carbon 17 of dihydrotestosterone. This suggests that tethers anchored to the 3′ nitrogen of **2f** may be expected to occupy the same cleft of the AR binding pocket as Danishefsky's testosterone conjugate tethers. To further support the validity of Miller's PR model, Carrondo et al, have examined the crystal structure of the LBDs of both human AR and PR bound to the steroidal ligand metribolone.[54] Comparison of the two crystal structures shows not only remarkable overlap of the amino acids defining the respective binding pockets, but also a nearly identical orientation of the bound ligand in both receptors. Figure 2 shows a space-filled rendering of metribolone bound to the human AR.[54] Of note is the fact that the ligand is buried within the receptor and is afforded little solvent exposure, a fact which, upon initial examination, proved to be quite discouraging. However, the ability of the receptor to tolerate modifications of the ligand, such as those shown in Figure 1, suggested that an appropriate tether may be expected to protrude into the cytosol from the LBD. On the basis of these observations, a fifth targeting group was synthesized, incorporating the alkynyl moiety employed by Danishefsky.

Scheme 2 shows the stepwise synthesis of the targeting group incorporating 2-butyne-1,4-diol in place of the ethylene glycol tethers. Reductive etherification of **3** with decaborane in the presence of molten 2-butyne-1,4-diol yields the corresponding benzylic ether **4c**. After removal of excess butynediol by repeated extraction, the crude product was dissolved in acetone where it was refluxed with 2,2-dimethoxypropane and a catalytic amount of *p*-toluenesulfonic acid to yield 70% of the benzoxazine-protected intermediate **5c** after two steps. Attempts to mesylate the alcohol, as was done with the ethylene glycol derivatives, gave a mixture of products consisting of primarily the desired, yet unstable, mesylate and the corresponding chlorinated product in varying ratios depending on the conditions used and the reaction time. The chlorinated product apparently results from displacement of the successfully installed methanesulfonate ester by the chloride ion liberated from consumed methanesulfonyl chloride. In an attempt to improve upon the yield and selectivity achieved in the introduction of a leaving group to the propargylic position of the tether, the mesylation reaction was repeated in the presence of 10 equiv of LiBr. This served to completely brominate the terminus of the tether, in 87% yield, which rendered **6c** as a superior substrate

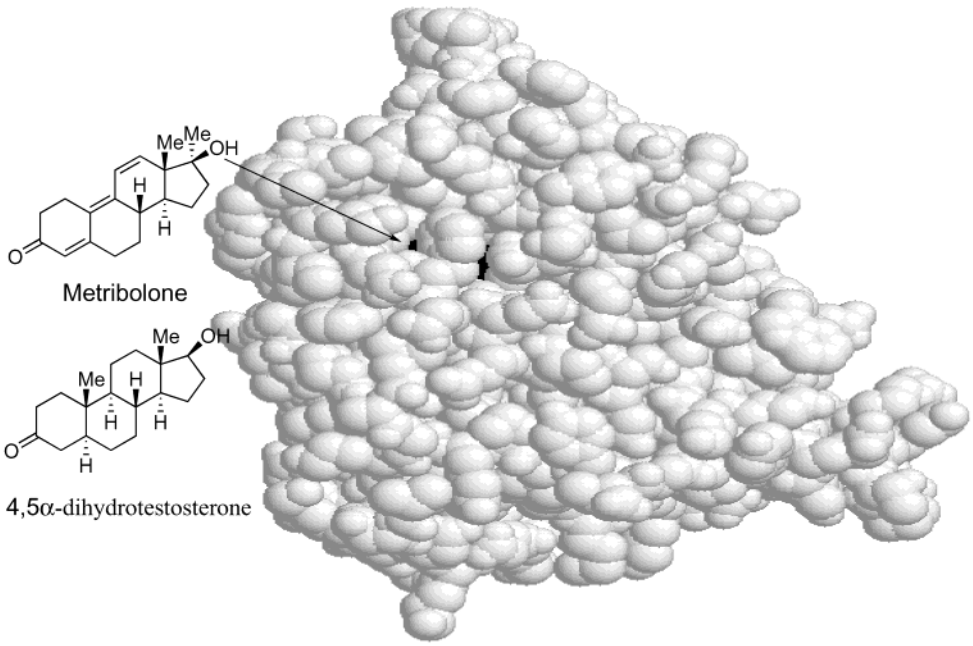


Figure 2. Space-filling model of the steroidal androgen metribolone (black) bound to the human androgen receptor (white). The buried nature of the 17-hydroxy moiety (black) of Metribolone illustrates the tight steric demands of the AR binding pocket. X-ray crystal structure reproduced from the Rutgers protein database entry 1E3G.[54]

for subsequent reaction with the **2f** anion. Displacement of the bromide with the sodium salt of **2f** gave the protected product **7c** in 82% yield. Finally, removal of the benzoxazine protecting group was carried out in 80% yield to give the desired compound **8c**.

Receptor Binding. The androgen receptor (AR) was obtained from PC3 cells (donated by Dr. Kerry Burnstein, University of Miami; Miami, FL) which had been stably transfected with the human androgen receptor cDNA (PC3/AR).[55] PC3/AR cells have been thoroughly characterized and have been shown to express the AR at ~ 596 fmol/mg total cellular protein, which is comparable to the expression of a mutant AR in the established LNCaP cell line (~816 fmol/mg).[56] PC3/AR cells were grown to near confluence, sonicated, and centrifuged to consistently yield 5.0 mL of a lysate containing approximately 1.9 mg/mL total cellular protein. Division of the collected lysate into 100 μL fractions yielded approximately 113 fmol of AR per aliquot (~ 1.1 nM). Crude lysate was used as the binding reaction medium in order to account for undesirable yet specific ligand−protein interactions. While purified AR can be used for the binding assay, we felt it was necessary to identify any unwanted binding events which supersede the affinity of the targeting compounds for the AR.

Work by Wakeling et al. suggests that nonsteroidal antiandrogens are characterized by rapid association and dissociation with the AR.[57] The Wakeling group found that while 30 min incubation in a steroidal radioligand competition assay led to efficient nonsteroidal ligand−receptor interactions, 18 h incubation showed dramatically decreased competition for binding. This phenomenon is proposed to reflect the rapid on/off rates for the nonsteroidal ligands as compared to the slow, tight binding affinity of steroids. This observation bodes well for our targeting scheme, as rapid association of our ligands with the AR is necessary in order to allow for efficient localization of doxsaliform. Likewise, rapid release of the ligand serves to liberate receptors of spent targeting molecules, which potentially allows for the acquisition and delivery of multiple prodrugs by a single receptor.[35]

A second concern may also be addressed by the rapid release of nonsteroidal ligands from the AR. The genetic instability and heterogeneous nature of neoplastic growths allows for varying expression patterns of gene products among cells within a single tumor.[58] Resistant tumors can, therefore, be seen to arise from selection for clones which do not express a targeted protein. While the rapid association of AR targeted prodrugs will serve to concentrate the cytotoxin within AR-expressing cells of a tumor, the rapid dissociation rate may allow for distribution of the prodrug into nonexpressing cells as well. Thus, the emergence of resistant colonies may be retarded by localized diffusion within the tumor.

The competitive binding assays were run for 30 min incubation periods to demonstrate the interaction of the nonsteroidal antiandrogens with AR during a relevant time frame for targeting. Tritiated Miboloerone (^{3}H-MIB) was chosen as the radioligand on account of its availability and extensive use in this capacity in the literature.[56,59] All assays were run at 4 °C, to avoid proteolytic degradation of the receptor, using a modified protocol which employs hydroxyapatite to sequester and wash the protein fraction of the assay solution.[60] Hydroxyapatite was supplied as an insoluble calcium phosphate coated agarose gel, which served to efficiently remove proteins from solution. The gel was then collected via filtration and washed extensively to remove background radioactivity due to nonspecific interactions with ^{3}H-MIB. Scintillation counting of the dry, washed gel and filter was then employed to quantify total binding of 1.0 nM ^{3}H-MIB in the presence of various concentrations of the test compounds. These numbers were then compared to controls for nonspecific binding and unchallenged total binding.[59] Results are shown in Table 1. All assays were performed in duplicate and

Table 1. IC_{50} and Relative Binding Affinity (RBA) Values of Test Ligands[a]

test compound	IC_{50} (nM)	RBA
nilutamide	9	100
2f	6	150
8a	77	12
8b	332	3
8c	49	18
12a	>1000	<1
12b	346	3
13	90	10
flutamide	154	6
salicylamide	≫1000	≪1

[a] IC_{50} and relative binding affinity values determined from competitive binding for the human AR of the various test ligands against 1.0 nM ^{3}H-Mibolerone in PC3/AR cell lysate at 4 °C.

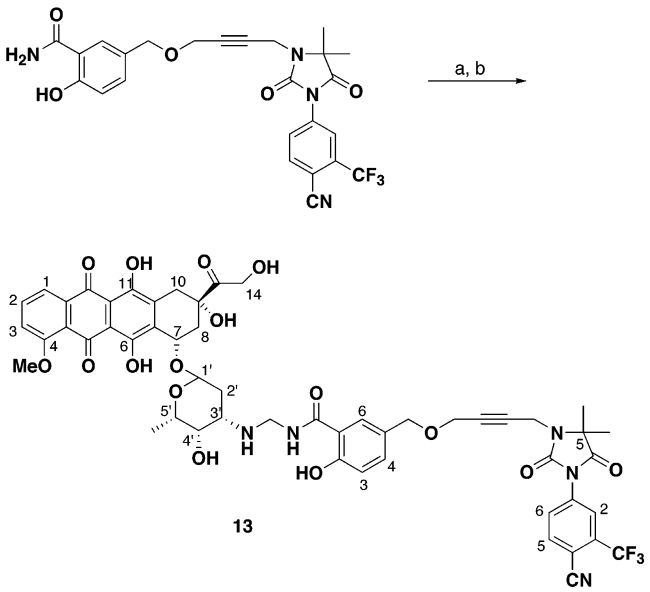
scintillation counting was repeated three times to ensure reproducibility of the data.

A concern with the development of a modified method is the validity relative to known protocols. There was an initial concern that the small differences in specific and nonspecific binding would not be accurately quantified by scintillation counting. To address this issue, a positive control using unlabled Mibolerone as the test ligand was performed. The cold Mibolerone was found to compete off 50% of the radioligand at a concentration of approximately 2.0 nM, suggesting that the developed method is a valid measure of competitive binding. Likewise, a negative control experiment was conducted using salicylamide, which is expected to show no specific binding to the AR. Table 1 shows that salicylamide was, in fact, ineffective at competing for AR binding in the presence of ^{3}H-Mibolerone. Further control experiments using the cytosolic fraction of PC3/neo cells, which do not express the androgen receptor,[56] also showed no specific binding of ^{3}H-Mibolerone, suggesting that the differences measured in PC3/AR lysate are real and AR specific.

Relative binding affinities (RBA) for the 10 analyzed compounds are listed in Table 1. The clinically employed nilutamide was found to inhibit ^{3}H-Mibolerone with an apparent IC_{50} of 10 nM and has been assigned an arbitrary RBA of 100. The RBAs of the other test compounds are expressed as fractions of nilutamide binding, based on their respective IC_{50} values. The RBA of **2f** (150%) suggests that the use of this molecule as the core of our targeting constructs was quite appropriate. The majority of the compounds tested exhibit RBA values between 1% and 20% of that observed for nilutamide, indicating that the introduction of our tethers has a detrimental effect on binding. However, the IC_{50} value of the best of the targeting groups, **8c** at 49 nM, is still on the same order of magnitude as the unmodified **2f**.

The tri(ethylene glycol) derivative **8b**, having the longest tether of the tested compounds, displayed only 3% of the binding affinity of nilutamide. This surprising finding is likely the result of the excessive flexibility of the tri(ethylene glycol) tether which is proposed to facilitate folding of the molecule and subsequent intramolecular interactions which preclude efficient receptor binding.[53] Also of interest is the poor ability of the piperazine analogues **12a** and **12b** to effectively displace ^{3}H-Mibolerone binding in the tested concentration range. The added steric demands of the piperazine ring or the presence of a cationic amine in the tether may account for this lack of activity.

Scheme 4[a]



[a] (a) Formalin, DMF, 55 °C, 15 min; (b) doxorubicin hydrochloride.

The di(ethylene glycol) and butynediol derivatives, **8a** and **8c**, respectively, exhibited the best RBA values, although they were only 12% and 18% as efficient as nilutamide, respectively, in competing for AR binding against ^{3}H-Mibolerone. Due to the short length of the tethers in these compounds, it is possible that the salicylamide moiety of each is responsible for beneficial interactions which improve the binding affinity. Finally, the alkynyl tether of **8c** apparently serves to maintain rigidity and direct the salicylamide portion of the molecule out from the binding pocket, much as it is proposed to do for Danishefsky's geldanamycin conjugates.[20]

Having identified compound **8c** as a potential targeting molecule, we proceeded to prepare the N-Mannich base which results from the condensation of **8c** with doxorubicin and formaldehyde as is shown in Scheme 4. The N-Mannich base was isolated in 60% yield and was found to compete with H^3-Mibolerone for AR binding with an affinity (IC_{50} = 90 nM) which was comparable to that of unmodified **8c** (Table 1**).** To address the concern of hydrolysis of **13** in the binding reaction, a solution of the targeted prodrug was prepared in the reaction buffer and incubated at 4 °C for 30 min. Reverse phase HPLC analysis indicated no appreciable hydrolysis of the N-Mannich base under these conditions, suggesting that the specific binding observed was attributed to **13** and not liberated **8c**. These results suggest that doxorubicin−formaldehyde conjugates, and perhaps various other cytotoxins, may be efficiently targeted to AR-expressing cells via attachment to nonsteroidal antiandrogens by a suitable tether. Future work will explore the AR interaction of these constructs in whole cells and the efficacy of the targeted N-Mannich base in a prostate tumor-expressing mouse model.

Several experiments were carried out in attempts to quantitate the cytotoxicity of **13**, relative to doxorubicin

and untargeted doxsaliform, against PC3/AR and PC3/neo cells. Experiments were run in cell culture media supplemented with either fetal bovine serum (FBS) or dextran-charcoal stripped calf serum in an attempt to account for the presence of testosterone in the unadulterated FBS. Unfortunately, an underlying problem prevented the accurate analysis of the effect of prodrug targeting. Extended treatment of cells with varying concentrations of the two prodrugs leads to release of the doxorubicin−formaldehyde conjugate by **13** and by untargeted doxsaliform, both inside and outside the cells, irrespective of receptor binding. Thus both prodrugs serve to bathe the cells in the doxorubicin−formaldehyde conjugate via hydrolysis over the course of the exposure period. Prodrug treatment times > 3 h were required for extensive hydrolysis of the N-Mannich base, but over this time period, both **13** and doxsaliform release the same amount of the doxorubicin−formaldehyde conjugate. An in vivo system is expected to allow for accumulation of the targeted prodrug in AR-expressing cells, where hydrolysis of the N-Mannich base will lead to localized delivery of the active drug. This should greatly contrast the deposition of the untargeted doxsaliform, which is expected to experience no preferential distribution. Simply stated, the targeted prodrug was designed to exploit a dynamic system of circulation, accumulation due to receptor binding, and release of the cytotoxin from an inactive conjugate, while cell culture only offers a static model for determining cytotoxicity. While these IC_{50} studies indicated that the potency of the targeted drug was not diminished relative to doxorubicin or the untargeted N-Mannich base prodrug, the effect of AR binding and subsequent release of the doxorubicin−formaldehyde conjugate could not be ascertained through cell culture experiments. However, preliminary fluorescence microscopy has shown that both **8c** and **13** do, in fact, bind to the AR in live cultured cells (work in progress). On the basis of the AR binding affinity of **13**, as determined here in cell lysate as well as in whole cells (data not shown), we are currently developing a mouse model, employing orthotopicly implanted prostate tumors, which will serve as a dynamic test system for assessment of the efficacy of **13**.

Experimental Section

General Remarks. Melting points were determined in open capillary tubes with a Thomas-Hoover capillary melting point apparatus and are uncorrected. ^{1}H NMR spectra were acquired with a Varian Unity Inova 500 MHz spectrometer. Unambiguous NMR assignments for the protons of the **2f**, salicylamide, and doxorubicin portions of the synthesized compounds are designated by "nil", "sal", or "dox", respectively. Mass spectral data were acquired on a VG Instruments AutospecM mass spectrometer by electron impact (EI) using a perfluorokerosene internal standard for [M+] data or liquid SIMS (LSIMS) ionization with a poly(ethylene glycol) (PEG) internal standard for [MH+] data. Mass spectral data for compound **13** were collected by Dr. Chris Hadad (Ohio State University; Columbus, OH) with a 3-Tesla Finnigan FTMS-2000 Fourier Transform mass spectrometer. HPLC analyses were performed with a Hewlett-Packard 1090 liquid chromatograph equipped with a diode array UV−vis detector and workstation; chromatography was performed with a Hewlett-Packard 5 μm reverse phase C_{18} microbore column, 2.1 mm i.d. x 100 mm, eluting at 0.5 mL/min, monitoring at 260 and 310 nm. Acceptable analytical resolution was achieved with gradients of acetonitrile and triethylammonium acetate (Et_3-NHOAc; TEAA), prepared as 20 mM triethylamine adjusted to pH 6.0 with acetic acid. The method employed for all analytical chromatography was as follows: A = CH_3CN, B = pH 6.0 buffer; A:B, 0:100 to 70:30 at 10 min, isocratic until 12 min, 0:100 at 15 min. For preparative HPLC, a 5 μm spherical particle C_{18} Ranin Dynamax semipreparative column was employed, 10 mm × 25 cm with a 10 mm × 5 cm guard column, eluting at 3.0 mL/min, monitoring at 260 and 310 nm. Adequate preparative separation was achieved using the following method: A = CH_3CN, B = 20 mM triethylamine adjusted to pH 3.5 or 4.0 as indicated with glacial acetic acid (TEAA buffer); A:B, 0:100 to 70:30 at 20 min, isocratic until 30 min, 0:100 at 35 min. Water was distilled and purified with a Millipore Q-UF Plus purification system to 18 Mohm-cm. Flash silica gel (particle size: 32−63 μm, pore size: 60 Å, cat. # 02826−25) was obtained from Scientific Adsorbants Inc. (Atlanta, GA). Labetalol hydrochloride (97.5%) was purchased from either Sigma (Milwaukee, WI) or Spectrum Chemicals (New Brunswick, NJ). Bis(2-bromoethyl) ether (96%) and 4-fluoro-2-(trifluoromethyl)benzonitrile (98%) were obtained from Lancaster Research Chemicals, (Windham, NH). Sodium periodate (reagent grade) and 5,5-dimethylhydantoin (97%) were purchased from Acros Organics (Pittsburgh, PA). Methanesulfonyl chloride (99.5%), *p*-toluenesulfonic acid (98.5%), decaborane (white), di(ethylene glycol) (99%), tri(ethylene glycol) (99%), 2-bromoethanol (95%), 2-butyne-1,4-diol (99%), 1,4-dibromobutane (99%), 2,2-dimethoxypropane (98%), and piperazine (99%) were all purchased from Aldrich (Milwaukee, WI). The BSA micro protein determination kit was purchased from Sigma (Milwaukee, WI), and the Complete-mini protease inhibitor cocktail was obtained from Roche. All other solvents and reagents were purchased from Fisher as reagent grade and used without further purification except for Fisher HPLC grade acetonitrile and Aldrich Sure-Seal (anhydrous) *N*,*N*-dimethylformamide (99.8%) and pyridine (99.8%).

All tissue culture materials were obtained from Gibco Life Technologies (Grand Island, NY) unless otherwise noted. PC3/AR and PC3/neo cells were a gift from Dr. Kerry L. Burnstein (University of Miami, FL). Both cell lines were maintained in vitro by serial culture in RPMI 1640 media supplemented with either 10% fetal bovine serum (Gemini Bio-Products, Calbasas, CA) or 10% dextran-charcoal stripped (delipidated) calf serum, L-glutamine (2 mM), HEPES buffer (10 mM), penicillin (100 units/mL), and streptomycin (100 μg/mL). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Phenol red-free RPMI 1640 media supplemented with L-glutamine was obtained from Sigma (Milwaukee, WI). Spin-X centrifuge filters were purchased from Costar/Corning (Corning, NY).

Syntheses. 4-(4,4-Dimethyl-2,5-dioxo-imidazolidin-1-yl)-2-trifluoromethylbenzonitrile (2f). To a stirring solution of 1.00 g (4.78 mmol) of 4-fluoro-2-(trifluoromethyl)-benzonitrile in 15.0 mL of DMF was added 3.10 g (23.9 mmol) of 5,5-dimethylhydantoin and 0.990 g (7.17 mmol) of K_2CO_3. The resulting suspension was stirred under an argon atmosphere at 55 °C for 16 h and then at 65 °C for 48 h. The reaction mixture was diluted to 300 mL with ethyl acetate, vacuum filtered, and rotary evaporated at 40 °C followed by 50 °C and 50 μm Hg to yield a bright yellow paste. The paste was dissolved in 25% hexanes/75% ethyl acetate and eluted from a silica gel flash column (35 cm × 3 cm) with 50% hexanes/50% ethyl acetate. The collected product was rotary evaporated at 40 °C to give a white solid which was recrystallized from ethyl acetate/hexanes to give 0.780 g (60%) of **2f** as a white crystalline solid (mp 208−210 °C): ^{1}H NMR (500 MHz, $(CD_3)_2$-CO) δ 1.53 (6H, s, 4-$(CH_3)_2$), 7.81 (1H, bs, NH), 8.13 (1H, dd, J = 8, 2 Hz, 5), 8.20 (1H, d, J = 8 Hz, 6), 8.25 (1H, d, J = 2 Hz, 3); *m*/*z* 297.0723 [M+] (calculated for 297.0725); anal. ($C_{13}H_{10}F_3N_3O_2$) C, H, N.

5-Formyl-2-hydroxybenzamide (3). A 600 mL stirring aqueous solution of 2.00 g (5.48 mmol) of Labetalol hydrochloride in a 1.0 L round-bottom flask was neutralized with 4 mL of saturated $NaHCO_3$. The reaction flask was then fitted with

a dropping funnel containing 1.17 g (5.48 mmol) of sodium periodate in 50 mL of Millipore H_2O. Dropwise addition of the periodate solution over 15 min at room temperature gave a pale pink solution which was stirred for an additional 20 min. The solution was acidified with 3.0 mL of concentrated aqueous HCl and stirred vigorously until a white precipitate was formed (approximately 2 min). The resulting suspension was stored for 12 h at 4 °C, to facilitate precipitation, at which time it was filtered. The collected solid was recrystallized from 80 mL of boiling Millipore H_2O and allowed to sit for 12 h at 4 °C. Vacuum filtration gave 0.634 g (70%) of **3** as white to pale golden needles (mp 204−206 °C): ^{1}H NMR (500 MHz, $(CD_3)_2CO$) δ 7.06 (1H, d, J = 8 Hz, 3), 7.47 (1H, bs, NH), 7.98 (1H, dd, J = 8, 2 Hz, 4), 8.33 (1H, bs, NH), 8.40 (1H, d, J = 2 Hz, 6), 9.84 (1H, s, HCO), 13.87 (1H, s, 2-OH); *m/z* 165.0421 [M+] (calculated for 165.0426).

2-Hydroxy-5-[2-(2-hydroxyethoxy)ethoxymethyl]benzamide (4a). A solution of 200 mg (1.21 mmol) of **3** was prepared in 10 mL of di(ethylene glycol) heated under an argon atmosphere to 70 °C in a mineral oil bath. After dissolution was complete, the solution was removed from the oil bath and allowed to cool for 5 min at which time 74 mg (0.61 mmol) of decaborane was added. Strong effervescence was observed over 5 min but then subsided. The reaction was then placed back in the oil bath and was stirred at 70 °C for 5 h. The solvent was removed by rotary evaporation at 60 °C and 50 μm Hg. After the bulk of the solvent was removed, the remaining oil was transferred to a separatory funnel with 300 mL of ethyl acetate. This solution was washed 4× with 50 mL portions of saturated brine and the organic layer was collected, dried over anhydrous magnesium sulfate, and rotary evaporated at 40 °C to a pale yellow oil. The desired product was then collected from a silica gel flash column (35 cm × 3 cm diameter), eluting with 10% hexanes/90% ethyl acetate. Removal of the solvent by rotary evaporation at 40 °C gave 291 mg (91%) of **4a** as a clear, colorless oil: ^{1}H NMR (500 MHz, $(CD_3)_2CO$ δ 3.49−3.54 (2H, m, OCH_2CH$_2$OH), 3.55−3.59 (2H, m, 1/2(OCH$_2$CH$_2$O)), 3.59−3.66 (4H, m, CH_2OH, 1/2(OCH$_2$CH$_2$O)), 3.89 (1H, bs, CH$_2$OH), 4.44 (2H, s, Bn), 6.86 (1H, d, J = 9 Hz, 3), 7.19 (1H, bs, NH), 7.4 (1H, dd, J = 9, 2 Hz, 4), 7.81 (1H, d, J = 2 Hz, 6), 8.01 (1H, bs, NH), 12.9 (1H, bs, 2-OH); *m/z* 255.1106 [M+] (calculated for 255.1107).

6-[2-(2-Hydroxyethoxy)ethoxymethyl]-2,2-dimethyl-2,3-dihydro-benzo[*e*][1,3]oxazin-4-one (5a). A sample of 200 mg (0.78 mmol) of **4a** was dissolved in 20 mL of acetone and 10 mL of 2,2-dimethoxypropane. A catalytic amount of *p*-toluenesulfonic acid was added, and the resulting solution was refluxed under an argon atmosphere at 80 °C for 1.5 h. The solvent was then removed by rotary evaporation at 40 °C. The resulting brown residue was transferred to a separatory funnel in 250 mL of ethyl acetate and was washed 3× with 50 mL portions of saturated brine containing 5% K_2CO_3. The organic layer was collected, dried over anhydrous magnesium sulfate, and rotary evaporated at 40 °C to give a yellow oil. The washed product was then eluted from a silica gel flash column (35 cm × 3 cm) in 5% hexanes/95% ethyl acetate. Removal of solvent yielded 204 mg (88%) of pure **5a** as a clear, colorless oil: ^{1}H NMR (500 MHz, $(CD_3)_2CO$) δ 1.61 (6H, s, 2-(CH$_3$)$_2$), 3.50−3.53 (2H, m, OCH_2CH$_2$OH), 3.59−3.65 (6H, m, CH_2OH, OCH$_2$CH$_2$O), 3.73 (1H, t, J = 6 Hz, CH$_2$OH), 4.52 (2H, s, Bn), 6.92 (1H, d, J = 9 Hz, 8), 7.47 (1H, dd, J = 9, 2 Hz, 7), 7.82 (1H, bs, NH), 7.85 (1H, d, J = 2 Hz, 5); *m/z* 295.1412 [M+] (calculated for 295.1420).

6-{2-[2-(2-Hydroxyethoxy)ethoxy]ethoxymethyl}-2,2-dimethyl-2,3-dihydro-benzo[*e*][1,3]oxazin-4-one (5b). A solution of 200 mg (1.21 mmol) of **3** was prepared in 10 mL of tri(ethylene glycol) heated to 70 °C under an argon atmosphere in a mineral oil bath. After dissolution was complete, the solution was removed from the oil bath and allowed to cool for 5 min before 74 mg (0.60 mmol) of decaborane was added. Strong effervescence was observed over 5 min but then subsided. The reaction was then placed back in the oil bath and was stirred at 70 °C for 5 h. The solvent was then removed by heating to 125 °C in a Kügelrohr oven at 150 μm Hg for 2 h. After the bulk of the solvent was removed, the remaining viscous liquid was transferred to a separatory funnel with 100 mL of saturated brine. This solution was extracted into 4 × 200 mL portions of ethyl acetate, which were collected, pooled, dried over anhydrous magnesium sulfate, and rotary evaporated at 40 °C to a pale yellow oil. The desired, semipure product **4b** was then collected from a silica gel flash column (35 cm × 3 cm), eluting with 5% hexanes/95% ethyl acetate. This semipure product was dissolved in 20 mL of acetone and 10 mL of 2,2-dimethoxypropane. A catalytic amount of *p*-toluenesulfonic acid was added, and the resulting solution was refluxed at 80 °C for 1.5 h. The solvent was then removed by rotary evaporation at 40 °C. The resulting brown residue was transferred to a separatory funnel in 300 mL of ethyl acetate and was washed 3× with 50 mL portions of saturated brine containing 5% K_2CO_3. The organic layer was collected, dried over magnesium sulfate, and rotary evaporated to a pale yellow oil at 40 °C. The washed product was then eluted from a silica gel flash column (35 cm × 3 cm) in 5% methanol/95% ethyl acetate. Removal of solvent yielded 231 mg (56% in two steps) of pure **5b** as a clear, colorless oil: ^{1}H NMR (500 MHz, $(CD_3)_2CO$) δ 1.61 (6H, s, 2-(CH$_3$)$_2$), 3.50−3.53 (2H, m, OCH_2-CH$_2$OH), 3.56−3.65 (10H, m, OCH$_2$CH_2OH, 2(OCH$_2$CH$_2$O)), 3.74−3.77 (1H, m, CH$_2$OH), 4.51 (2H, s, Bn), 6.92 (1H, d, J = 8 Hz, 8), 7.48 (1H, dd, J = 8, 2 Hz, 7), 7.84 (1H, d, J = 2 Hz, 5), 7.99 (1H, bs, NH); *m/z* 339.1681 [M+] (calculated for 339.1682).

6-(4-Hydroxybut-2-ynyloxymethyl)-2,2-dimethyl-2,3-dihydro-benzole[*e*][1,3]-oxazin-4-one (5c). A solution of 200 mg (1.21 mmol) of **3** was prepared in 10 g of 1,4-butyne-2-diol by heating a mixture of the two solids at 70 °C in a mineral oil bath for 15 min. The resulting solution was removed from the oil bath and allowed to cool for 5 min before 74 mg (0.60 mmol) of decaborane was added. Strong effervescence was observed for approximately 5 min but then subsided. The reaction was then stirred under an argon atmosphere at 60 °C for 5 h and was subsequently diluted to 300 mL with ethyl acetate and transferred to a separatory funnel. After 8 × 40 mL washes with saturated brine, the organic layer was collected, dried over anhydrous magnesium sulfate, and rotary evaporated at 40 °C to give approximately 1.5 mL of a clear amber oil. The crude product was diluted to 10 mL in ethyl acetate and was eluted from a silica gel flash column (30 cm × 2 cm) in 100% ethyl acetate. Rotary evaporation of the solvent gave **4c** as a semipure clear, pale yellow oil. The product from the flash column was dissolved in 20 mL of acetone and 10 mL of 2,2-dimethoxypropane. A catalytic amount of *p*-toluenesulfonic acid was added and the resulting solution was refluxed at 85 °C for 1.5 h. The solvent was removed by rotary evaporation and the resulting oil was transferred to a separatory funnel in 300 mL of ethyl acetate. This solution was washed 3x with 40 mL portions of saturated brine containing 5% K_2CO_3 and the organic layer was collected, dried over anhydrous magnesium sulfate and rotary evaporated at 40 °C to give 233 mg (70% in two steps) of **5c** as a clear, colorless oil: ^{1}H NMR (500 MHz, $(CD_3)_2CO$) δ 1.62 (6H, s, 2-(CH$_3$)$_2$), 3.45 (1H, bs, CH$_2$OH), 4.17 (2H, bm, CCH_2OH), 4.3 (2H, bm, OCH_2C), 4.53 (2H, s, Bn), 6.68 (1H, 2, J = 8 Hz, 8), 7.43 (1H, dd, J = 8, 2 Hz, 7), 7.90 (1H, d, J = 2 Hz, 5), 8.21 (1H, bs, NH); *m/z* 275.1145 [M+] (calculated for 275.1158).

Methanesulfonic Acid 2-[2-(2,2-Dimethyl-4-oxo-3,4-dihydro-2*H*-benzo[*e*][1,3]oxazin-6-ylmethoxy)ethoxy]ethyl Ester (6a). To a stirring solution of 200 mg (0.68 mmol) of **5a** in 4 mL of THF were added 55 μL (0.68 mmol) of dry pyridine and 160 μL (2.1 mmol) of methanesulfonyl chloride. This solution was stirred under an argon atmosphere at room temperature for 30 min at which time 380 μL (2.8 mmol) of triethylamine was added. A white precipitate was formed immediately and the reaction was stirred for 2 h at room temperature. The reaction mixture was then diluted to 300 mL with ethyl acetate and transferred to a separatory funnel. After 3 × 40 mL washes with saturated brine containing 5% NaH_2PO_4, the organic layer was collected, dried over anhydrous magnesium sulfate, and rotary evaporated at 40 °C to

a pale yellow oil. This crude oil was dissolved in 8 mL of ethyl acetate and introduced to a silica gel flash column (35 cm × 3 cm) packed in 10% hexanes/90% ethyl acetate. Elution with the same, followed by removal of solvent by rotary evaporation at 40 °C, gave 233 mg (92%) of **6a** as a clear, colorless oil: ^{1}H NMR (500 MHz, $(CD_3)_2CO$ δ 1.62 (6H, s, 2-$(CH_3)_2$), 3.1 (3H, s, SO_2CH_3), 3.62−3.66 (2H, m, 1/2(OCH_2CH_2O)), 3.67−3.70 (2H, m, 1/2(OCH_2CH_2O)), 3.73−3.78 (2H, m, OCH_2CH_2OMs), 4.34 − 4.39 (2H, m, OCH_2CH_2OMs), 4.52 (2H, s, Bn), 6.92 (1H, d, J = 9 Hz, 8), 7.48 (1H, dd, J = 9, 2 Hz, 7), 7.74 (1H, bs, NH), 7.81 (1H, d, J = 2 Hz, 5); *m*/*z* 373.1188 [M+] (calculated for 373.1195).

Methanesulfonic Acid 2-{2-[2-(2,2-dimethyl-4-oxo-3,4-dihydro-2*H*-benzo [*e*][1,3]oxazin-6-ylmethoxy)ethoxy]-ethoxy}ethyl Ester (6b). 6b was prepared as **6a** in 90% yield: ^{1}H NMR (500 MHz, $(CD_3)_2CO$ δ 1.61 (6H, s, 2-$(CH_3)_2$), 3.10 (3H, s, SO_2CH_3), 3.58−3.65 (8H, m, 2(OCH_2CH_2O)), 3.75 (2H, m, OCH_2CH_2OMs), 4.35 (2H, m, OCH_2CH_2OMs), 4.51 (2H, s, Bn), 6.92 (1H, d, J = 9 Hz, 8), 7.48 (1H, dd, J = 9, 2 Hz, 7), 7.83 (1H, d, J = 2 Hz, 5), 7.92 (1H, bs, NH); *m*/*z*: 417.1452 [M^+] (calculated for 417.1452).

6-(4-Bromobut-2-ynyloxymethyl)-2,2-dimethyl-2,3-dihydro-benzo[*e*][1,3]oxazin-4-one (6c). To a stirring solution of 150 mg (0.55 mmol) of **5c** in 2.0 mL of THF were added 45 μl (0.55 mmol) of pyridine and 170 μl (2.2 mmol) of methanesulfonyl chloride. After 30 min stirring under argon, 380 μL (2.7 mmol) of triethylamine was added, and the resulting solution was allowed to stir for 1 h at room temperature, during which time a white precipitate gradually formed. To this stirring suspension was added 480 mg (5.5 mmol) of LiBr as a solution in 2 mL of THF. Once HPLC indicated completion of the reaction, the resulting suspension was diluted to 200 mL with ethyl acetate and was washed 3× with 40 mL portions of saturated brine containing 5% NaH_2PO_4. The organic layer was then collected, dried over anhydrous magnesium sulfate, and rotary evaporated at 40 °C to give a pale yellow oil. The washed product was eluted from a silica gel flash column (30 cm × 2 cm) in 10% hexanes/90% ethyl acetate. Removal of solvent by rotary evaporation at 40 °C yielded 162 mg (87%) of **6c** as a clear, colorless oil: ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.67 (6H, s, 2-$(CH_3)_2$), 3.95−3.98 (2H, m, OCH_2C), 4.21−4.25 (2H, m, CCH_2Br), 4.55 (2H, s, Bn), 6.21 (1H, d, J = 8 Hz, 8), 7.47 (1H, dd, J = 8, 2 Hz, 7), 7.89 (1H, d, J = 2 Hz, 5), 8.08 (1H, s, NH); *m*/*z* 337.0314 [M+] (calculated for 337.0314).

5-(2-{2-[3-(4-Cyano-3-trifluoromethylphenyl)-5,5-dimethyl-2,4-dioxo-imidazolidin-1-yl]ethoxy}ethoxymethyl)-2-hydroxybenzamide (8a). A solution of 100 mg (0.34 mmol) of **2f** was prepared in 2.0 mL of DMF, and to this was added 13.5 mg (0.34 mmol) of sodium hydride as a 60% emulsion in oil. The mixture was stirred under an argon atmosphere at room temperature for 3 h. The resulting yellow solution was then added to a stirring solution of 127 mg (0.34 mmol) of **6a** in 2.0 mL of DMF, and the reaction flask was heated to 60 °C in a mineral oil bath for 24 h. The product was precipitated by dropwise addition of the reaction mixture to 100 mL of saturated aqueous NaH_2PO_3. The pale yellow precipitate was then extracted into 250 mL of ethyl acetate, which was collected and rotary evaporated at 40 °C to yield a yellow solution of crude product in DMF. Further rotary evaporation at 50 °C and 100 μm Hg removed the DMF to give a viscous yellow oil. This residue was eluted from a silica gel flash column (30 cm × 2 cm) in 10% hexanes/90% ethyl acetate to yield semipure **7a**. The fractions containing **7a** which were collected from the column were pooled and the solvent removed via rotary evaporation at 40 °C to give a clear, colorless oil which was dissolved in 10 mL of 80% methanol/20% water. A catalytic amount of *p*-toluenesulfonic acid was added, and the resulting solution was refluxed at 90 °C for 30 h. The methanol was removed and 50 mL of saturated brine was used to transfer the resulting emulsion to a separatory funnel where it was extracted into 250 mL of ethyl acetate and washed 2× with 40 mL portions of saturated brine containing 5% $NaHCO_3$. Collection of the organic layer, followed by drying over anhydrous magnesium sulfate and rotary evaporation at 40 °C, gave a pale yellow oil. Elution from a silica gel flash column (30 cm × 2 cm) in 10% hexanes/90% ethyl acetate yielded 82 mg (42% in two steps) of **8a** as a clear, colorless oil: ^{1}H NMR (500 MHz, $(CDCl_3)$ δ 1.54 (6H, s, nil-5-$(CH_3)_2$), 3.54−3.59 (2H, m, OCH_2CH_2N), 3.61−3.65 (2H, m, 1/2(OCH_2CH_2O)), 3.66−3.70 (2H, m, 1/2(OCH_2CH_2O)), 3.74−3.79 (2H, m, OCH_2CH_2N), 4.46 (2H, s, Bn), 5.91 (1H, bs, NH), 6.62 (1H, bs, NH), 6.95 (1H, d, J = 8 Hz, sal-3), 7.33 (1H, dd, J = 8, 2 Hz, sal-4), 7.41 (1H, d, J = 2 Hz, sal-6), 7.88 (1H, d, J = 9 Hz, nil-5), 7.92 (1H, dd, J = 9, 2 Hz, nil-6), 8.09 (1H, d, J = 2 Hz, nil-2), 12.22 (1H, bs, 2-OH); *m*/*z* 534.1711 [M+] (calculated for 534.1726); anal. ($C_{25}H_{25}F_3N_4O_6$) C, H, N.

5-[2-(2-{2-[3-(4-Cyano-3-trifluoromethylphenyl)-5,5-dimethyl-2,4-dioxo-imidazolidin-1-yl]ethoxy}ethoxy)ethoxymethyl]-2-hydroxybenzamide (8b). 8b was prepared as **8a** in 58% yield: ^{1}H NMR (500 MHz, $(CD_3)_2CO$) δ 1.57 (6H, s, nil-5-$(CH_3)_2$), 3.58−3.61 (4H, m, OCH_2CH_2O), 3.61−3.67 (6H, m, OCH_2CH_2N, OCH_2CH_2O), 3.73−3.76 (2H, m, OCH_2CH_2N), 4.46 (2H, s, Bn), 6.90 (1H, d, J = 8 Hz, sal-3), 7.19 (1H, bs, NH), 7.45 (1H, dd, J = 8, 2 Hz, sal-4), 7.81 (1H, d, J = 2 Hz, sal-6), 7.97 (1H, bs, NH), 8.17 (1H, dd, J = 9, 2 Hz, nil-6), 8.22 (1H, d, J = 9 Hz, nil-5), 8.29 (1H, d, J = 2 Hz, nil-2), 12.95 (1H, s, 2-OH); *m*/*z* 578.2000 [M+] (calculated for 578.1988); anal. ($C_{27}H_{29}F_3N_4O_7$) C, H, N.

4-{3-[4-(2,2-Dimethyl-4-oxo-3,4-dihydro-2*H*-benzo[*e*][1,3]-oxazin-6-ylmethoxy)but-2-ynyl]-4,4-dimethyl-2,5-dioxo-imidazolidin-1-yl}-2-trifluoromethylbenzonitrile (7c). A solution of 130 mg (0.44 mmol) of **2f** was prepared in 2.0 mL of DMF, and to this was added 18 mg (0.45 mmol) of sodium hydride as a 60% emulsion in oil. The mixture was stirred under an argon atmosphere at room temperature for 3 h. The resulting yellow solution was then added to a stirring solution of 150 mg (0.44 mmol) of **6c** in 2.0 mL of DMF. The reaction was then stirred for 6 h at room temperature under argon. The product was precipitated by dropwise addition of the reaction mixture to 100 mL of saturated aqueous NaH_2PO_3. The pale yellow precipitate was then extracted into 300 mL of ethyl acetate which was collected and rotary evaporated at 40 °C to yield a yellow solution of crude product in DMF. Further rotary evaporation at 50 °C and 100 μmHg removed the DMF to give a viscous yellow oil. This residue was eluted from a silica gel flash column (30 cm × 2 cm) in 10% hexanes/90% ethyl acetate. Removal of solvent by rotary evaporation gave 200 mg (82%) of pure **7c** as a pale yellow oil: ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.64 (12H, s, sal-2-$(CH_3)_2$, nil-4-$(CH_3)_2$), 4.16−4.19 (2H, m, OCH_2C), 4.29−4.33 (2H, m, CCH_2N), 4.53 (2H, s, Bn), 6.91 (1H, d, J = 8 Hz, sal-8), 7.44 (1H, dd, J = 8, 2 Hz, sal-7), 7.53 (1H, bs, NH), 7.87 (1H, d, sal-5), 7.21 (1H, d, J = 8 Hz, nil-6), 8.00 (1H, dd, J = 8, 2 Hz, nil-5), 8.15 (1H, d, J = 2 Hz, nil-3); *m*/*z* 554.1757 [M+] (calculated for 554.1777).

5-{4-[3-(4-Cyano-3-trifluoromethylphenyl)-5,5-dimethyl-2,4-dioxo-imidazolidin-1-yl]but-2-ynyloxymethyl}-2-hydroxybenzamide (8c). A solution of 200 mg of **7c** (0.36 mmol) was prepared in 10 mL of 20% water/80% MeOH, and a catalytic amount of *p*-toluenesulfonic acid was added. The reaction was refluxed under an argon atmosphere for 24 h at 90 °C. The solvent was then removed by rotary evaporation at 40 °C, and the residue was transferred to a separatory funnel in 200 mL of ethyl acetate. This solution was washed 2× with 40 mL portions of saturated brine containing 5% $NaHCO_3$. Th organic layer was then collected, dried over anhydrous magnesium sulfate, and rotary evaporated at 40 °C to give a pale yellow oil. Elution from a silica gel flash column (30 cm × 2 cm) in 5% hexanes/95% ethyl acetate followed by rotary evaporation yielded a product of approximately 95% purity. Further purification by preparatory HPLC, eluting with 20 mM pH 4.0 TEAA buffer, yielded 148 mg (80%) of **8c** as a clear colorless oil: ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.63 (6H, s, nil-5-$(CH_3)_2$), 4.15 (2H, s, OCH_2C), 4.29 (2H, s, CCH_2N), 4.50 (2H, s, Bn), 5.90 (1H, bs, NH), 6.62 (1H, bs, NH), 6.96 (1H, d, J = 9 Hz, sal-3), 7.37 (1H, dd, J = 9, 2 Hz, sal-4), 7.46 (1H, d, J = 2 Hz, sal-6), 7.92 (1H, d, J = 8 Hz, nil-5), 7.97 (1H, dd, J = 8, 2 Hz, nil-6), 8.11 (1H, d, J = 2 Hz,

nil-2), 12.30 (1H, s, 2-OH); *m/z* 514.1469 [M+] (calculated for 514.1464); anal. ($C_{25}H_{21}F_3N_4O_5$) C, H, N.

5-(2-Bromoethoxymethyl)-2-hydroxybenzamide (9). A solution of 150 mg (0.91 mmol) of **3** was prepared in 12 mL of 2-bromoethanol by heating a stirring mixture of the two to 55 °C under an argon atmosphere. The solution was then allowed to cool for 5 min at room temperature before 56 mg (0.46 mmol) of decaborane was added. Excessive evolution of H_2 was observed for 5 min, after which time the reaction was again heated to 55 °C. After being stirred for 4 h, the solvent was removed by rotary evaporation at 40 °C and 100 μmHg. The residue was dissolved in 8 mL of ethyl acetate and introduced to a silica gel flash column (30 cm × 2 cm) packed in 25% hexanes/75% ethyl acetate. Elution with the same solvent system yielded 175 mg (70%) of **9** as a clear, colorless oil: ^{1}H NMR (500 MHz, $(CD_3)_2CO$) δ 3.55 (2H, t, J = 6 Hz, OCH_2-CH_2Br), 3.76 (2H, t, J = 6 Hz, OCH_2CH_2Br), 4.47 (2H, s, Bn), 6.89 (1H, d, J = 8 Hz, 3), 7.19 (1H, bs, NH), 7.44 (1H, dd, J = 8, 2 Hz, 4), 7.81 (1H, d, J = 2 Hz, 6), 7.97 (1H, bs, NH), 12.93 (1H, s, 2-OH); *m/z* 273.007 [M+] (calculated for 273.001).

4-[3-(4-Bromo-butyl)-4,4-dimethyl-2,5-dioxo-imidazolidin-1-yl]-2-trifluoromethylbenzonitrile (10a). To a stirring solution of 306 mg (1.0 mmol) of **2f** in 3.0 mL of DMF was added 49 mg (1.2 mmol) of sodium hydride (60% in oil). The resulting suspension was stirred at room temperature for 1.5 h at which time evolution of H_2 had ceased and a yellow solution persisted. To this solution was added 1.0 mL of 1,4-dibromobutane, and the resulting reaction mixture was heated under an argon atmosphere to 60 °C for 0.5 h. At this time, the reaction mixture was added dropwise to 100 mL of saturated brine containing 5% NaH_2PO_4. A pale yellow precipitate was formed which was extracted into 250 mL of ethyl acetate. The organic layer was washed 2× with saturated brine, collected, and rotary evaporated at 40 °C to a yellow solution in DMF. Further rotary evaporation at 50 °C and 100 μmHg removed the DMF to yield a yellow oil. This crude product was dissolved in 10 mL of 50% hexanes/50% ethyl acetate and introduced to a silica gel flash column (30 cm × 2 cm) packed in 75% hexanes/25% ethyl acetate. Elution with 75% hexanes/25% ethyl acetate followed by removal of solvent by rotary evaporation at 40 °C gave 378 mg (85%) of **10a** as a clear, colorless oil: ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.54 (6H, s, 4-$(CH_3)_2$), 1.82−1.98 (2H, m, CH_2CH_2N), 1.82−1.98 (2H, m, $BrCH_2CH_2$), 3.36−3.42 (2H, m, CH_2CH_2N), 3.43−3.49 (2H, m, $BrCH_2CH_2$), 7.90 (1H, d, J = 8 Hz, 6), 7.99 (1H, dd, J = 8, 2 Hz, 5), 8.14 (1H, d, J = 2 Hz, 3); *m/z* 431.0450 [M+] (calculated for 431.0456).

4-{3-[2-(2-Bromoethoxy)ethyl]-4,4-dimethyl-2,5-dioxo-imidazolidin-1-yl}-2-trifluoromethylbenzonitrile (10b). 10b was prepared as **10a** in 82% yield: ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.55 (6H, s, 4-$(CH_3)_2$), 3.47 (2H, m, OCH_2CH_2N), 3.56 (2H, m, OCH_2CH_2N), 3.76, (2H, m, $BrCH_2CH_2O$), 3.81 (2H, m, $BrCH_2CH_2O$), 7.91 (1H, d, J = 9 Hz, 6), 8.00 (1H, dd, J = 9, 2 Hz, 5), 8.14 (1H, d, J = 2 Hz, 3); *m/z* 447.0405 [M+] (calculated for 447.0405).

4-{4,4-Dimethyl-2,5-dioxo-3-[2-(2-piperazin-1-ylethoxy)ethoxy]imidazolidin-1-yl}-2-trifluoromethylbenzonitrile (11b). 11b was prepared as **11a** in 59% yield: ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.48 (6H, s, 4-$(CH_3)_2$), 2.45−2.60 (4H, bm, $(CH_2)_2NCH_2$), 2.51−2.56 (2H, m, $(CH_2)_2NCH_2$), 2.86−2.98 (4H, bm, $HN(CH_2)_2$), 3.46−3.53 (2H, m, CH_2OCH_2), 3.55 (2H, t, J = 6 Hz, CH_2NCO), 3.61−3.66 (2H, m, CH_2OCH_2), 5.20 (1H, bs, NH), 7.87 (1H, d, J = 8 Hz, 6), 7.95 (1H, dd, J = 8, 2 Hz, 5), 8.09 (1H, d, J = 2 Hz, 3); *m/z* 452.1897 [M−H] (calculated for 452.1909).

5-[2-(4-{4-[3-(4-Cyano-3-trifluoromethyl-phenyl)-5,5-dimethyl-2,4-dioxo-imidazolidin-1-yl]butyl}piperazin-1-yl)ethoxymethyl]-2-hydroxybenzamide (12a). To a stirring solution of 120 mg (0.27 mmol) of **11a** and 80 mg (0.29 mmol) of **9** in 2.0 mL of THF was added 100 μL (0.72 mmol) of triethylamine. The resulting solution was refluxed under an argon atmosphere for 20 h, at which time the solvent was removed by rotary evaporation at 40 °C. The residue was dissolved in 250 mL of ethyl acetate and transferred to a separatory funnel where it was washed 3× with 40 mL portions of saturated brine containing 5% NaH_2PO_4. The organic layer was collected, dried over anhydrous magnesium sulfate, and rotary evaporated free of solvent at 40 °C to give a pale brown oil. This residue was dissolved in 10 mL of ethyl acetate and introduced to a silica gel flash column (20 cm × 2 cm) packed in the 100% ethyl acetate. Elution with 78% ethyl acetate/20% methanol/2% triethylamine followed by removal of the solvent by rotary evaporation at 40 °C gave a pale golden oil. Further purification by preparatory HPLC using 20 mM pH 4.0 TEAA buffer was required and yielded 121 mg (71%) of **12a** as a clear, colorless oil: ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.49−1.60 (2H, m, $(CH_2)_2NCH_2CH_2$), 1.52 (6H, s, 5-$(CH_3)_2$), 1.67−1.76 (2H, m, CH_2CH_2NCO), 2.37 (2H, t, J = 7 Hz, $(CH_2)_2$-NCH_2CH_2), 2.49 (8H, bs, $N(CH_2CH_2)_2N$), 2.61 (2H, t, J = 6 Hz, $BnOCH_2CH_2N$) 3.36 (2H, t, J = 8 Hz, CH_2NCO), 3.56 (2H, t, J = 6 Hz, $BnOCH_2$), 4.42 (2H, s, Bn), 6.23 (1H, bs, NH), 6.87 (1H, bs, NH), 6.91 (1H, d, J = 8 Hz, sal-3), 7.32 (1H, d, J = 8 Hz, sal-4), 7.48 (1H, s, sal-6), 7.90 (1H, d, J = 9 Hz, nil-5), 7.99 (1H, dd, J = 9, 2 Hz, nil-6), 8.14 (1H, d, J = 2 Hz, nil-2); *m/z* 631.2833 [MH+] (calculated for 631.2856).

5-{2-[4-(2-{2-[3-(4-Cyano-3-trifluoromethyl-phenyl)-5,5-dimethyl-2,4-dioxo-imidazolidin-1-yl]ethoxy}-ethylpiperazine-1-yl]ethoxymethyl}-2-hydroxybenzamide (12b). 12b was prepared as **12a** in 83% yield: ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.53 (6H, s, 5-$(CH_3)_2$), 2.45−2.68 (8H, bm, $N(CH_2CH_2)_2N$), 2.54−2.61 (2H, m, $(CH_2)_2NCH_2CH_2O$), 2.63−2.90 (2H, m, $BnOCH_2CH_2N$), 3.50−3.63 (6H, m, $BnOCH_2$, CH_2-OCH_2CH_2NCO), 3.65−3.71 (2H, m, OCH_2CH_2NCO), 4.43 (2H, s, Bn), 6.13 (1H, bs, NH), 6.92 (1H, d, J = 8 Hz, sal-3), 7.31 (1H, dd, J = 8, 2 Hz, sal-4), 7.57 (1H, d, J = 2 Hz, sal-6), 7.90 (1H, d, J = 8 Hz, nil-5), 8.00 (1H, dd, J = 8, 2 Hz, nil-6), 8.14 (1H, d, J = 2 Hz, nil-2); *m/z* 647.2816 [MH+] (calculated for 647.2805).

***N*-(5-{4-[3-(4-Cyano-3-trifluoromethylphenyl)-5,5-dimethyl-2,4-dioxo-imidazolidin-1-yl]but-2-ynyloxymethyl)-2-hydroxybenzamidomethyl)doxorubicin (13).** To a stirring solution of 20 mg of **8c** (0.04 mmol) in 2.0 mL of DMF was added 10 μL of a 37% formalin solution (0.13 mmol). The reaction was stirred in a screw top vial for 15 min at 55 °C, at which time 20 mg (0.03 mmol) of doxorubicin hydrochloride was added to form a red suspension which was stirred at 55 °C. After 15 min, a clear red solution had formed and the reaction was removed from the heat. Transfer of the solution to a 250 mL round-bottom flask, followed by rotary evaporation of the solvent at 55 °C and 50 μmHg gave a red film which was readily dissolved in 20 mL of methanol containing 30% of 20 mM pH 1.9 1% TFA. After 10 min at room temperature, the methanol was removed by rotary evaporation at 30 °C, and the resulting aqueous suspension was diluted to 100 mL with saturated brine and transferred to a separatory funnel. Extraction into 50 mL of chloroform followed by addition of 1 mL of glacial acetic acid and rotary evaporation at 30 °C gave a red film. The product was then dissolved in 1−2 mL of methanol and filtered through a 0.45 μm Spin-X centrifuge filter. Purification was achieved by preparative HPLC using a pH 3.5 TEAA buffer as the aqueous eluent. Pure material was collected into a test tube (100 mm × 10 mm) containing 0.5 mL of 1.0 M HCl. Acetonitrile was removed by rotary evaporation at 30 °C to yield an aqueous suspension of the pure product which was diluted to 50 mL with saturated brine and transferred to a separatory funnel. Extraction into 50 mL of chloroform followed by addition of 1 mL of glacial acetic acid and rotary evaporation at 30 °C gave 23 mg (60%) of **13** as the acetate salt: ^{1}H NMR (500 MHz, $(CD_3)_2CO$) δ 1.32 (3H, d, J = 6 Hz, dox-5′-CH_3), 1.62 (6H, s, 5-$(CH_3)_2$), 2.15−2.30 (3H, m, 2(dox-2′), dox-8), 2.42 (1H, d, J = 14 Hz, dox-8), 2.93 (1H, d, J = 18 Hz, dox-10), 3.12 (1H, J = 18 Hz, dox-10), 3.85−4.0 (1H, bm, dox-3′), 4.06 (3H, s, dox-4-OCH_3), 4.12 (3H, s, dox-9OH, CCH_2NCO), 4.38 (5H, s, dox-5′, Bn, $BnOCH_2C$), 4.62−4.78 (2H, m, dox-14), 4.91 (1H, bs, NCH_2N), 5.03 (1H, bs, NCH_2N), 5.21 (1H, s, dox-7), 5.56 (1H, s, dox-1′), 6.71 (1H, d, J = 8 Hz, sal-3), 7.29 (1H, d, J = 8 Hz, sal-4), 7.63 (1H, d, J = 8 Hz, dox-3), 7.74 (1H, bs, sal-6), 7.90 (1H, t, J = 8 Hz, dox-

2), 7.96 (1H, d, $J = 8$ Hz, dox-1), 8.14 (1H, d, $J = 8$ Hz, nil-5), 8.20 (1H, d, $J = 9$ Hz, nil-6), 8.25 (1H, s, nil-2), 10.32 (1H, bs, NH), 11.82 (1H, bs, sal-2-OH), 13.26 (1H, s, dox-6/11-OH), 14.18 (1H, s, dox-6/11-OH); *m/z* 1092.3101 [MNa+] (calculated for 1092.3097).

Radioligand Competition AR Binding Assay. PC3/AR or PC3/neo cells were grown in RPMI 1640 medium to approximately 80% confluency in five Nunc T-175 flasks. Growth medium in each flask was then replaced with 50 mL of phenol red-free RPMI 1640 supplemented with 10% dextran-coated charcoal-stripped FBS, and the cells were grown for an additional 18−22 h. Two hours prior to harvesting, the growth medium was again replaced with fresh phenol red-free, charcoal-stripped RPMI. The cells were then washed with 10 mL of Hank's balanced salt solution and dissociated with trypsin. Trypsin was quenched with phenol red-free, charcoal stripped RPMI, and the combined cells from each flask were centrifuged in a 50 mL conical tube at 100 x g for 5 min. The cells were then resuspended in 50 mL of phenol red-free, charcoal-stripped RPMI and counted at this concentration. Centrifugation at 100 x *g* gave approximately 1 mL of cells which were resuspended in 5 mL of 4 °C lysis buffer (10 mM Tris, 1.5 mM EDTA, 0.5 mM DTT, 10 mM $NaMoO_4$, 1.0 mM PMSF, 10% v/v glycerol) supplemented immediately before use with Complete-mini protease inhibitor cocktail. Cells were lysed via sonication at 4 °C with a microtip, set at maximum power, for 10 cycles of 6 s on and 24 s off. The cytosolic fraction of the lysate was isolated by ultracentrifugation at 4 °C and 225 000 x *g* for 45 min. The centrifuged samples were dispensed into 100 μL aliquots and stored at −78 °C until used. Total protein was quantified either in fresh or frozen aliquots by the Sigma BSA micro protein determination method according to the prescribed protocol.

Aliquots of cell lysate were used fresh or thawed at 4 °C. Stock solutions of 100x working concentration of the test ligands, ^{3}H-Mibolerone and unlabled Mibolerone were prepared in DMSO and subsequently diluted to 10× in lysis buffer. Concentrations of test compounds were determined spectrophotometricly in DMSO by either absorbance at 310 nm for salicylamide containing molecules ($\epsilon_{310} = 3580$ L/(mol × cm); as determined from a Beer−Lambert plot described by varying concentrations of **8a**), 264 nm for **2f** ($\epsilon_{264} = 13000$ L/(mol × cm)), or 276 nm for nilutamide ($\epsilon_{276} = 4620$ L/(mol × cm)). Aliquots of cell lysate were complemented with 10 μL of 10× ligand solutions and 10 μL of the 10× ^{3}H-Mibolerone solution to yield concentrations of 1, 10, 100, and 1000 nM test compound and 1 nM ^{3}H-Mibolerone. Each reaction was prepared in duplicate to yield eight total test assays. Duplicate positive controls, consisting of 10 μL of lysis buffer in place of a test ligand (total radioligand binding), and negative controls, consisting of 1000 nM unlabled Mibolerone (nonspecific binding), each in the presence of 1 nM ^{3}H-Mibolerone, were prepared. The reactions were gently mixed and briefly centrifuged before incubating at 4 °C for 30 min. After incubation was complete, 100 μL of each reaction was introduced to 400 μL of ice cold hydroxyapatite (HA), as a 60% suspension in pH 7.4 Tris buffer, on a 0.45 μm nylon filter in a Spin-X centrifuge tube. Upon addition of the reaction solution, the tubes were closed, briefly vortexed, and allowed to incubate on ice for 12 min with vortexing every 3−5 min. The HA suspensions were then centrifuged at 1200 x *g* for 10 min. The filtrate was discarded and the dry pellet was resuspended in 400 μL of pH 7.3 20 mM Tris wash buffer containing 0.1% Triton-X100. Following seven rounds of resuspension and subsequent centrifugation, the final filtrate was discarded, and the dry pellet was centrifuged for an additional 15 min. The pellet and filter bucket for each sample were then transferred to 20 mL scintillation vials and 4 mL of scintillation cocktail was added to each. Vortexing for 30 s thoroughly mixed the pellet with the scintillation liquid before counting. Each sample was counted for five repetitions of 3 min counts. This counting protocol was then repeated two additional times to ensure precision. Specific binding for each test concentration was determined by subtracting the nonspecific binding control from the total binding determined for each concentration. Comparison to the specific binding for the positive control, in which no competing ligand was incubated with the ^{3}H-Mibolerone, yielded the percent of ^{3}H-Mibolerone displaced by a given concentration of test ligand. The IC_{50} values for each test ligand were calculated by Logit-log(pseudo-Hill) analysis.

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Supporting Information Available: ^{1}H NMR spectra for all products and isolated intermediates; semilogarithmic IC_{50} determinations. This material is available free of charge via the Internet at http://pubs.acs.org.

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