Bioorganic & Medicinal Chemistry 18 (2010) 134–141

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Synthesis of 3-[(*N*-carboalkoxy)ethylamino]-indazole-dione derivatives and their biological activities on human liver carbonyl reductase

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ARTICLE INFO

Article history:
Received 5 September 2009
Revised 4 November 2009
Accepted 5 November 2009
Available online 10 November 2009

Keywords:
Carbonyl reductase
Indazole-dione
Pyrazoloquinone
Anthracycline therapy

ABSTRACT

A series of indazole-dione derivatives were synthesized by the 1,3-dipolar cycloaddition reaction of appropriate substituted benzoquinones or naphthoquinones and *N*-carboalkoxyamino diazopropane derivatives. These compounds were evaluated for their effects on human carbonyl reductase. Several of the analogs were found to serve as substrates for carbonyl reductase with a wide range of catalytic efficiencies, while four analogs display inhibitory activities with IC_{50} values ranging from 3–5 μM. Two of the inhibitors were studied in greater detail and were found to be noncompetitive inhibitors against both NADPH and menadione with K_I values ranging between 2 and 11 μM. Computational studies suggest that conformation of the compounds may determine whether the indazole-diones bind productively to yield product or nonproductively to inhibit the enzyme.

1. Introduction

Anthracyclines such as doxorubicin and daunorubicin (Fig. 1A) are anti-neoplastic agents commonly used to treat a variety of cancers.[1–5] However, the use of anthracyclines in treating cancer is significantly limited by a potentially lethal cardiotoxicity.[3–5] Formation of reactive oxygen species has been proposed as a major pathway for the cause and development of anthracycline-induced cardiotoxicity.[6] As a result, extensive studies and tests have been conducted to evaluate the cardio-protective effectiveness of antioxidants against anthracycline-induced cardiotoxicity, but these efforts have resulted in only limited success.[7,8] So far, the antioxidant and iron chelator dexrazoxane is the only drug approved by the FDA as an adjuvant therapy for protection against anthracycline-induced cardiotoxicity.[6,9] However, this drug offers only partial cardio-protection, and its use is accompanied by dose-dependent leukopenia.[7] Therefore, it is necessary to develop new and effective means of stemming anthracycline-induced cardiotoxicity based on mechanisms that are different from those involving the generation of reactive oxygen species. One promising strategy involves inhibiting a pathway by which anthracyclines are converted to alcohol metabolites.

Several reports suggest that the C13-hydroxy metabolites of the anthracyclines (Fig. 1) are the principal agents responsible for the observed cardiotoxicity.[10–17] Enzymes with NADPH-dependent carbonyl reductase activity are known to reduce the C13-carbonyl of anthracyclines to form the cardiotoxic hydroxyl metabolites. Specifically, several studies have correlated the concentrations of carbonyl reductase (CR; EC 1.1.1.184) with the risk of developing anthracycline-induced cardiotoxicity.[18,19] For example, decreased levels of CR from heterozygous mice for a null allele of a CR gene protected against the development of anthracycline cardiotoxity.[19] However, overexpression of CR in transgenic mice greatly increased susceptibility to anthracycline cardiotoxicity.[18]

It was also found that the parent anthracyclines, rather than the alcohol metabolites, are largely responsible for the anti-neoplastic

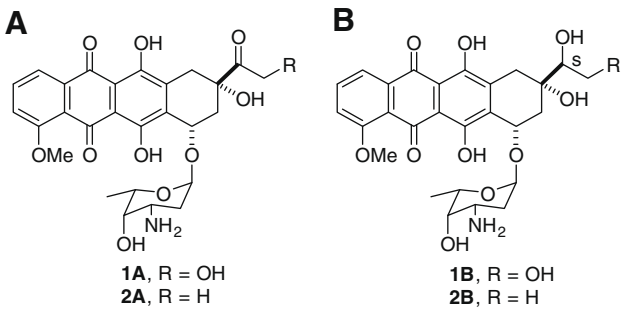

Figure 1. Structures of anthracyclines and C13-hydroxy metabolites. (A) Doxorubicin, R = OH and daunorubicin, R = H. (B) Doxorubicinol, R = OH and daunorubicinol, R = H.

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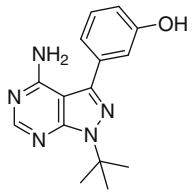
doi:10.1016/j.bmc.2009.11.011

properties,[11] while the cardiotoxic alcohol metabolites possess significantly reduced anticancer properties. Thus, anthracycline reduction has been postulated to be a mechanism for anthracycline drug resistance.[20] Since anthracycline reduction by CR leads to the formation of alcohol metabolites that are more cardiotoxic and less cytotoxic than the parent anthracyclines, it appears that anthracycline reduction by CR contributes to the cardiotoxicity problem in at least two ways: (1) It reduces the amount of anthracycline in circulation thereby necessitating an increase in the dose needed for effective treatment; (2) An increased anthracycline dosage results in an increase in the amount of cardiotoxic metabolite formed. Consequently, this increase in metabolite concentration increases the risk of cardiotoxicity. Such observations suggest that CR could be a pharmacological target for inhibitors that block its action in order to reduce the risk of cardiotoxicity associated with anthracycline therapy and improve anthracycline anticancer efficacy.

Numerous compounds have been found to inhibit CR,[21,22] with flavonoids such as rutin, quercetin, and quercitrin being among the most potent inhibitors.[21] Tanaka et al.[23] have recently reported the pyrazolopyrimidine 3-(1-*tert*-butyl-4-amino-1*H*-pyrazolo[3,4-*d*]pyrimidin-3-yl)phenol (**3**) of the Src family kinase inhibitors as a potent inhibitor of CR-catalyzed NADPH-dependent reduction of menadione to menadiol (IC_{50} = 788 nM). In order to study the efficacy of CR inhibition on anthracycline efficacy, they prepared an analog which showed poor inhibition against protein kinases but maintained potent inhibition against CR (IC_{50} = 759 nM). This potent and selective inhibitor of CR, 3-(7-isopropyl-4-(methylamino)-7*H*-pyrrolo[2,3-*d*]pyrimidin-5yl)phenol (**4**), was able to effect a 25% enhancement of daunorubicin-mediated A549-cell killing, consistent with its ability to inhibit CR-mediated metabolism of daunorubicin.[23]



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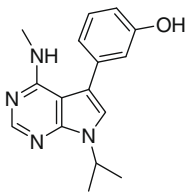

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While investigating the anticancer properties of novel indazole-dione derivatives, we were prompted to evaluate the biological activities of these compounds on the CR pathway. Our screening revealed this new class of indazole-diones to be inhibitors and substrates of CR, and we herein report the synthesis of several 3-[(*N*-carboalkoxy)ethylamino]indazole-dione derivatives and their biological activities on human CR.

2. Results and discussion

2.1. Chemistry

The indazole-diones in this work were prepared as outlined in Scheme 2. Nitrosation of tetrahydro-2-pyrimidone furnished *N*-nitrosotetrahydro-2-pyrimidone which reacted with alcoholic potassium alkoxides to generate 3-(*N*-carboalkoxyamino)-1-diazopropanes. The in situ cycloaddition of the diazopropane intermediates with appropriate benzoquinone and naphthoquinone derivatives in a mixture of THF/Et_2O at room temperature furnished the corresponding indazole-dione derivatives.[24] Some of the quinones used as dipolarophiles in the cycloaddition reaction were obtained commercially, but the phenylamino-1,4-benzoquinones were synthesized by adding a solution of aniline derivatives (**5**) in aqueous acetic acid to an aqueous solution of 1,4-benzoquinone (**6**) in an ice bath (Scheme 1) according to the method of Barakat et al.[25] This reaction is known to usually give a mixture of mono- and di-anilino-1,4-benzoquinones, but no purification was done to separate the two as only the monosubstituted derivatives react in a 1,3-dipolar cycloaddition reaction to give phenylamino indazole-diones.

In addition, the 1,3-dipolar cycloaddition of the diazopropane intermediates with almost all the quinone dipolarophiles was found to be regioselective and furnished only the 6-substituted indazole-dione. However, the cycloaddition of 3-(*N*-carbomethoxyamino)-1-diazopropane to 2-chloro-1,4-benzoquinone produced a regioisomeric mixture of 6- and 5-chloroindazol-4,7-dione (**19A** and **19B**, Scheme 3).

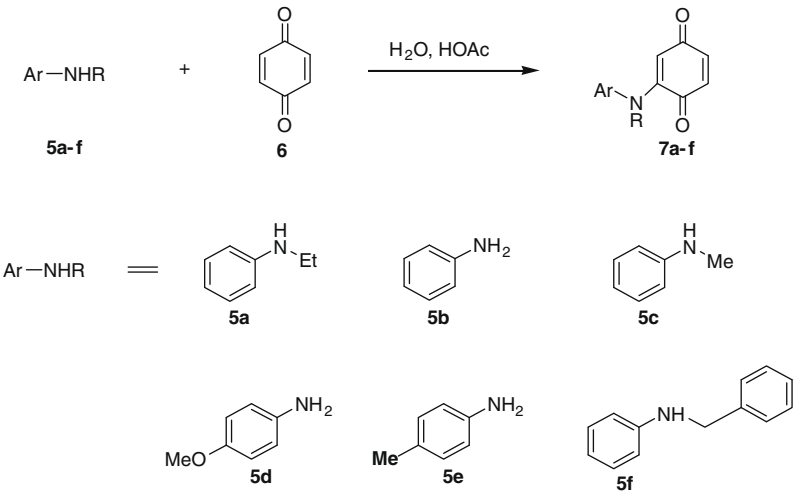
We have previously reported[24] the synthesis of compounds **20** and **21** while all the other analogs in this study are new.

2.2. Biological activities on carbonyl reductase

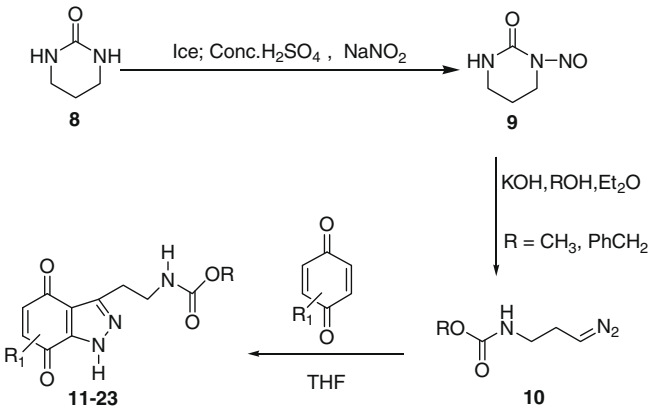
Quinones are known to be good substrates of carbonyl reductase.[21] Most of the indazole-dione derivatives in this study were also found to be substrates of CR with different catalytic efficiencies. Table 1 lists the different kinetic constants of CR for the different indazole-dione substrates. The k_{cat}/K_m, which is a measure of an enzyme's catalytic efficiency, varied by about 450-fold for CR and the indazole-dione substrates in this work. The substrates with the greatest catalytic efficiencies (ranging from 0.087 to 1.3 $\mu M^{-1}\ s^{-1}$) are indazole-diones **19A**, **20** and **21**, which have either a chlorine or a methoxy substituent at position 6 of the indazole-dione. The substrates with the lowest catalytic efficiencies (ranging from 0.0030 to 0.0051 $\mu M^{-1}\ s^{-1}$) are indazole-diones **14**–**16**, which have a phenyl amino substituent at position 6 of the indazole-dione. By way of comparison, menadione, an excellent CR substrate which is often used as a model quinone substrate, has a k_{cat}/K_m value of 0.098 $\mu M^{-1}\ s^{-1}$.[26] The differences in k_{cat}/K_m values between the best and worst substrates most likely results from a combination of electronic and steric factors. On one hand the phenyl amino substituents are far bulkier than the substituents present at position 6 in compounds **19A**, **20**, and **21** and, therefore, could interfere with binding interactions that promote efficient catalysis. On the other hand, electronic factors seem to underlie the differences in the catalytic efficiencies of compounds **19A** and **21**. Whereas compound **19A** (the best substrate) possesses an electron-withdrawing chlorine group at position 6, compound **21** possesses an electron-donating methoxy group at this position. The slight difference in the substrate efficiencies of compounds **20** and **21** further underscores the importance of steric contributions to the activities of these compounds upon interaction with the binding domain of CR. While both compounds **20** and **21** possess a methoxy substituent at position 6 of the indazole-dione scaffold, compound **20** incorporates a bulkier benzyl group (compared to the smaller methyl group of compound **21**) on the carbamate appendage making it a less efficient substrate than **21**.

Four indazole-dione derivatives (**11**, **12**, **22**, and **23**) were found to be inhibitors of CR with IC_{50} values of 3–5 μM against menadione and 11–25 μM against daunorubicin (Table 2). Further studies with indazole-diones **11** and **12** revealed that they are noncompetitive inhibitors against both NADPH and menadione (Table 3). Similar inhibition patterns were observed for the similarly structured pyrazolopyrimidine inhibitors.[23,27] Such a finding suggests that these inhibitors bind to several forms of the enzyme, as indicated in Figure 2.

The chemical structures of these inhibitors are only slightly different from those of the indazole-dione substrates discussed above. For example, among the benzene-fused indazole-diones, the substitution of hydrogen at position 8 of substrate **17** with a hydroxyl group gives the indazole-dione **23**, which is an inhibitor of CR. The small structural changes on the benzene-fused indazole-diones that give rise to the inhibitory activities may involve interactions, perhaps through hydrogen bonding with amino acids in the active site, that disrupt the proper positioning of the



Scheme 1.



Scheme 2.

substrate carbonyls for reduction by NADPH. In fact, X-ray structure analysis of CR with the pyrazolpyrimidine inhibitor **3** embedded in the binding site showed the hydroxyl group on **3** to make key H-bond interactions with some residues of the active site of CR.[23] A similar interaction with indazole-dione **23** may also be responsible for the inhibitory activity.

For indazole-diones with a phenylamino substituent, replacement of hydrogen attached to nitrogen of the phenylamino group by a methyl or ethyl group converted the indazole-dione derivative from a substrate to an inhibitor. In an attempt to explain why such a substitution converts a phenylamino-substituted indazole-dione from a substrate to an inhibitor, computational methods were employed. A comparison of the energy minimized structures for indazole-diones **11**, **12**, and **15** shows that the orientation of the phenyl ring with respect to the indazole-dione ring is markedly different when the amino hydrogen is replaced with either a methyl or an ethyl substituent (Fig. 3). For example, the phenyl ring of the substrate indazole-dione **15** is almost coplanar with the indazole-dione ring. However, substituting the hydrogen on the 6-amino group with an ethyl or methyl substituent (**11** and **12**, respectively) presumably induces unfavorable eclipsing interactions that cause the phenyl ring to twist perpendicular to the indazole-dione ring. This apparent difference in orientation could result in different binding modes for the substrate and inhibitor phenylamino indazole-diones such that the catalytic residues and NADPH are positioned properly for catalysis when the phenyl ring is more in plane with the indazole-dione ring. Further work is underway to gain additional insight into the structural requirements for a substrate versus an inhibitor of CR for this class of compounds.

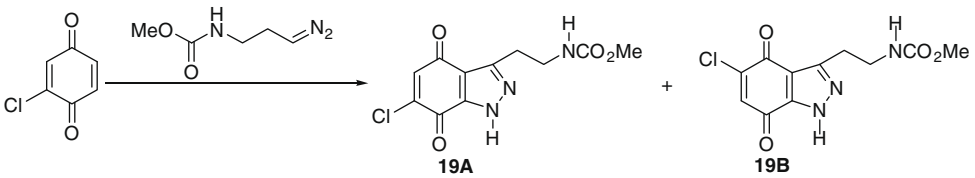
3. Conclusion

We have synthesized a small library of novel indazole-diones and assessed their biological activities on human carbonyl reductase (CR). Most of the indazole-diones were found to be substrates, while four analogs were found to be inhibitors of human CR with IC_{50} values of 3–5 μM. These inhibitors offer new leads and the potential to improve anthracycline therapy by reducing the cardiotoxicity associated with anthracyclines, while also increasing the anticancer efficacy of anthracyclines. More importantly, this study serves as a lead in a pursuit of the understanding of the structural requirements for substrate versus inhibitory effects on CR.

4. Experimental section

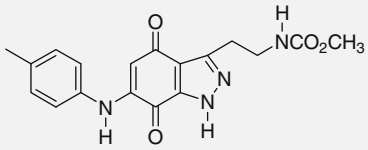
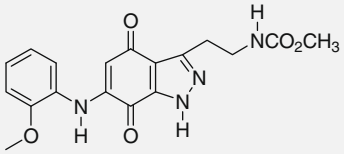
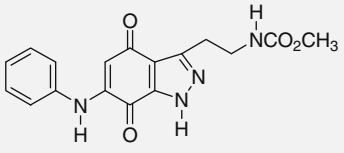
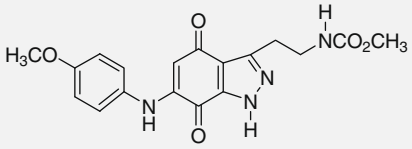
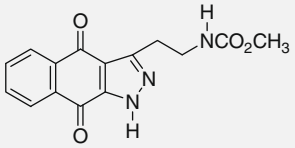
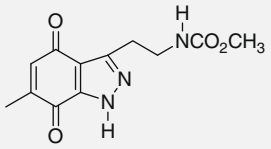
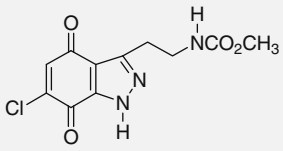
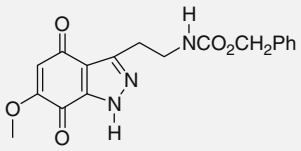
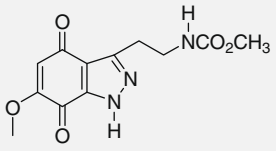
4.1. Chemistry: general procedures.

All reactions were carried out using laboratory grade materials and solvents. Purification by column chromatography was done using 70–230 mesh silica gel. Melting points were determined in



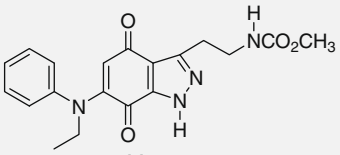
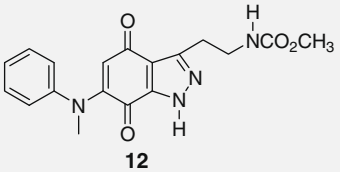
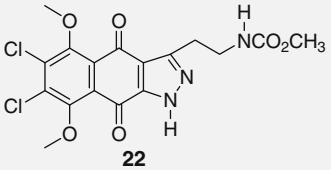
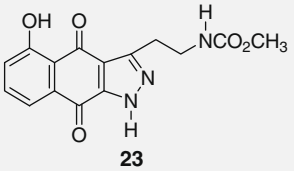
Scheme 3.

Table 1
Values of K_m, k_{cat}, and k_{cat}/K_m for indazole-dione substrates of carbonyl reductase

Compound	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}\ s^{-1}$)
 **13**	22 (4)	0.26 (0.02)	0.012 (0.001)
 **14**	25 (5)	0.073 (0.005)	0.0030 (0.0005)
 **15**	57 (4)	0.270 (0.009)	0.0048 (0.0001)
 **16**	27 (4)	0.14 (0.01)	0.0051 (0.0003)
 **17**	45 (4)	0.37 (0.016)	0.0082 (0.0004)
 **18**	130 (20)	0.72 (0.06)	0.0056 (0.0003)
 **19A**	0.76 (0.057)	1.0 (0.02)	1.3 (0.08)
 **20**	16 (2.5)	1.4 (0.08)	0.087 (0.009)
 **21**	5 (2)	0.91 (0.05)	0.17 (0.05)

open capillary tubes on a Mel-Temp melting point apparatus and are uncorrected. The IR spectra were performed in KBr on a Nicolet Magna 560 or a Nicolet Avatar 370 FTIR spectrometer. The ^{1}H NMR spectra were obtained on a Bruker Avance 400 MHz spectrometer in deuterated dimethyl sulfoxide (DMSO-d_6). Chemical shifts are in δ units (ppm) with $(CH_3)_2SO$ (2.50 ppm) as the internal standard.

Table 2
IC_{50} values of indazole-dione inhibitors on carbonyl reductase

Compound	IC_{50} versus menadione (μM)	IC_{50} versus daunorubicin (μM)
 **11**	4	13
 **12**	4	11
 **22**	5	25
 **23**	3	20

Peaks due to methylene protons α to the urethane group of the indazole-diones in this study are buried or overlap significantly with the water peak at 3.25–3.50 ppm. This was confirmed by obtaining the ^{1}H NMR spectrum of one indazole-dione (compound **11**) in deuterated chloroform ($CDCl_3$). The ^{1}H NMR spectrum of **11** in $CDCl_3$ displayed a 2H multiplet centered at 3.53 ppm for the methylene protons α to the urethane group, whereas, the peak for the same protons was buried in the water peak at 3.33 ppm in the DMSO-d_6 spectrum. For most of the compounds, mass spectral measurements were done using a Finnigan Navigator mass spectrometer; while for three compounds, high resolution mass spectra (EI or FAB) were recorded on a VG Analytical 70-SE mass spectrometer equipped with a 11–250 J data system. Methanol was used as the solvent, and all the mass spectra showed signals corresponding to the molecular ion plus sodium (Na). *N*-Nitrosotetrahydro-2(*H*)-pyrimidone was prepared as previously reported.[24]

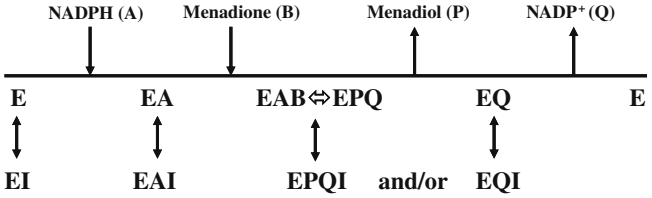


Figure 2. Kinetic mechanism of human carbonyl reductase with inhibitor binding. The kinetic mechanism for human carbonyl reductase is ordered bi–bi as indicated above. *E* is the enzyme, *A* is NADPH, *B* is menadione, *P* is menadiol, and *Q* is $NADP^+$. *I* represents the inhibitor indazole-diones **11** and **12**. Arrows pointing into the horizontal line indicate binding to enzyme and arrows pointing away indicate release from the enzyme.

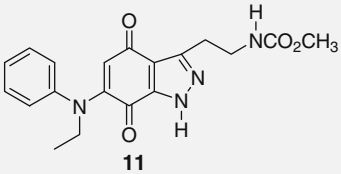
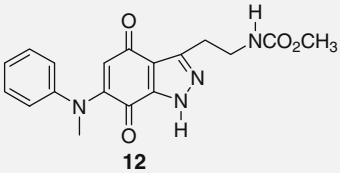
4.2. Synthesis of phenylamino-1,4-benzoquinones (7a–f) as exemplified by the synthesis of 2-(*N*-ethylphenylamino)-1,4-benzoquinone (7a)

Benzoquinone (**6**, 2.0884 g, 19.32 mmol) was dissolved in hot distilled water (200 mL) and the solution was filtered. The filtrate was cooled down to −8 °C, and a solution of *N*-ethylaniline (**5a**, 1.2 mL, 9.506 mmol) was added to it (the solution of *N*-ethylaniline was prepared by adding *N*-ethylaniline (1.2 mL) into water (75 mL) and then adding glacial acetic acid until all the *N*-ethylaniline dissolved). The resulting mixture was stirred at 0 °C for 15 min, and filtered under suction to yield a dark purple solid (0.7320 g) which was used without purification for the next experiment.

4.3. General procedure for the synthesis of indazole-diones with phenylamino substituents as exemplified by the synthesis of 3-(methyl-*N*-ethylcarbamate)-6-(*N*′-ethylphenylamino)indazol-4,7-dione (11)

N-Nitrosotetrahydro-2-pyrimidone (**9**, 0.7040 g, 5.4523 mmol) was added in small portions to a cooled mixture of KOH (1.0044 g) in methanol (10 mL) and diethyl ether (50 mL). The resultant mixture was stirred at room temperature for 25 min and washed with saturated NaCl solution (2 × 25 mL), 50% aqueous NaCl (2 × 25 mL), and saturated NaCl (2 × 25 mL). The ether layer was added to a solution of 2-(*N*-ethylphenylamino)-1,

Table 3
Inhibition constants for indazole-dione inhibitors **11** and **12**

Inhibitor	Fixed substrate (concentration)	Varied substrate (concentrations)	Kinetic constants (μM)
 **11**	NADPH (10 μM)	Menadione (10–200 μM)	K_{II} = 3.2 (0.14) K_{IS} = 11.3 (3.4)
	NADPH (50 μM)	Menadione (10–250 μM)	K_{II} = 3.3 (0.15) K_{IS} = 8.0 (1.8)
	NADPH (300 μM)	Menadione (5–130 μM)	K_{II} = 3.4 (0.17) K_{IS} = 5.2 (1.0)
	Menadione (50 μM)	NADPH (3–50 μM)	K_{II} = 2.5 (0.08) K_{IS} = 16.2 (7.8)
	Menadione (250 μM)	NADPH (3–50 μM)	K_{II} = 2.0 (0.16) K_{IS} = 2.3 (0.63)
 **12**	NADPH (10 μM)	Menadione (20–250 μM)	K_{II} = 2.9 (0.18) K_{IS} = 3.4 (0.83)
	NADPH (50 μM)	Menadione (14–250 μM)	K_{II} = 2.2 (0.21) K_{IS} = 7.3 (4.0)
	NADPH (300 μM)	Menadione (16–250 μM)	K_{II} = 4.6 (0.72) K_{IS} = 4.4 (4.2)

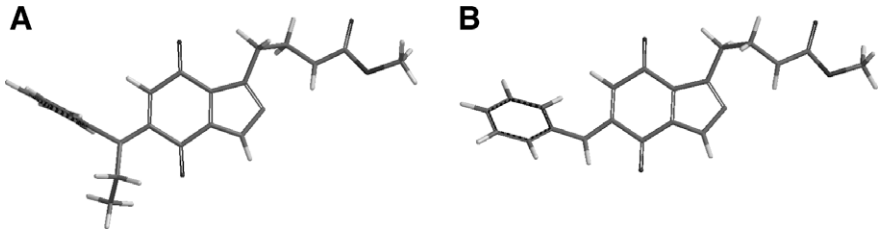


Figure 3. Energy minimized structures of indazole-diones **11** and **15**. (A) Indazole-dione **11** (inhibitor). (B) Indazole-dione **15** (substrate).

4-benzoquinone (**7a**, 0.6606 g, 2.9068 mmol) in THF (25 mL), and the resultant mixture was stirred at room temperature for 3 days. The mixture was concentrated in vacuo and diethyl ether (100 mL) was added to it. The resulting mixture was placed in the freezer overnight and filtered under suction to furnish a brick red solid of **11** (282.7 mg, 26.40%). Mp 95–96 °C. IR (cm^{-1}) 3336, 3121, 1697, 1615, 1548. ^1H NMR (DMSO-d_6) δ 1.10 (t, 3H, J = 6.5 Hz), 2.99 (t, 2H, J = 6.8 Hz), 3.51 (s, 3H), 3.78 (m, 2H), 5.84 (s, 1H), 7.13 (d, 2H, J = 7.5 Hz), 7.18–7.24 (m, 2H), 7.36 (t, 2H, J = 7.8 Hz), 14.00 (s, 1H). ESI MS m/z 391.0 ([M+Na]$^+$ calcd 391.1). ^1H NMR ($CDCl_3$) δ 1.23 (t, 3H, J = 7.0 Hz), 3.17 (dist. t, 2H), 3.53 (m, 2H), 3.63 (s, 3H), 3.84 (q, 2H, J = 7.0 Hz), 5.52 (br s, 1H), 5.82 (s, 1H), 7.10 (d, 2H, J = 8.0 Hz), 7.25 (t, 1H, J = 7.4 Hz), 7.37 (t, 2H, J = 7.73 Hz), 12.43 (br s, 1H).

4.3.1. 3-(Methyl-*N*-ethylcarbamate)-6-(*N*′-methylphenylamino) indazol-4,7-dione (12)

Mp 194–196 °C. IR (cm^{-1}) 3354, 3125, 1696, 1603, 1554. ^1H NMR (DMSO-d_6) δ 3.02 (t, 2H, J = 6.8 Hz), 3.52 (s, 3H), 5.93 (s, 1H), 7.13 (d, 2H, J = 7.6 Hz), 7.18 (t, 1H, J = 7.3 Hz), 7.27 (br t, 1H, J = 5.4 Hz), 7.34 (t, 2H, J = 7.8 Hz), 14.00 (s, 1H). ESI MS m/z 377.2 ([M+Na]$^+$ calcd 377.1).

4.3.2. 3-(Methyl-*N*-ethylcarbamate)-6-(*p*-methylphenylamino) indazol-4,7-dione (13)

Mp 212–214 °C. IR (cm^{-1}) 3335, 3250, 3118, 1696, 1624, 1591. ^1H NMR (DMSO-d_6) δ 2.31 (s, 3H), 3.00 (t, 2H, J = 7.0 Hz), 3.51 (s, 3H), 5.74 (s, 1H), 7.22 (s, 5H), 8.87 (s, 1H), 14.10 (s, 1H). FAB HRMS calcd for $C_{18}H_{19}N_4O_4$ m/z 355.1406 (M^++1), found 355.1440.

4.3.3. 3-(Methyl-*N*-ethylcarbamate)-6-(*o*-methoxyphenylamino) indazol-4,7-dione (14)

Mp 217–220 °C. IR (cm^{-1}) 3334, 3113, 1701, 1582. ^1H NMR (DMSO-d_6) δ 3.02 (t, 2H, J = 6.8 Hz), 3.51 (s, 3H), 3.85 (s, 3H), 5.54 (s, 1H), 7.03 (dt, 1H, J = 7.6, 1.3 Hz), 7.15 (dd, 1H, J = 8.3, 1.2 Hz), 7.20–7.28 (m, 2H), 7.33 (dd, 1H, J = 7.8, 1.5 Hz), 8.34 (s, 1H), 14.10 (s, 1H). EI HRMS calcd for $C_{18}H_{18}N_4O_5$ m/z 370.1277, found 370.1271.

4.3.4. 3-(Methyl-*N*-ethylcarbamate)-6-phenylaminoindazol-4,7-dione (15)

Mp 202–203 °C. IR (cm^{-1}) 3401, 3327, 3109, 1697, 1578. ^1H NMR (DMSO-d_6) δ 3.02 (t, 2H, J = 6.2 Hz), 3.51 (s, 3H), 5.83 (s, 1H), 7.17–7.21 (m, 1H), 7.24–7.27 (m, 1H), 7.35 (d, 2H, J = 7.4 Hz), 7.39–7.44 (m, 2H), 8.95 (s, 1H), 14.10 (s, 1H). ESI MS m/z 363.1 ([M+Na]$^+$ calcd 363.1).

4.3.5. 3-(Methyl-*N*-ethylcarbamate)-6-(*p*-methoxyphenylamino) indazol-4,7-dione (16)

Mp 191 °C (dec.). IR (cm^{-1}) 3324, 3222, 1696, 1613, 1571. ^1H NMR (DMSO-d_6) δ 3.00 (t, 2H, J = 6.9 Hz), 3.51 (s, 3H), 3.77 (s, 3H), 5.63 (s, 1H), 6.99 (d, 2H, J = 9.0 Hz), 7.22–7.31 (m, 3H), 8.86 (s, 1H), 14.10 (s, 1H). ESI MS m/z 393.2 ([M+Na]$^+$ calcd 393.1).

4.4. General procedure for the synthesis of indazole-diones without phenylamino substituents as exemplified by the synthesis of 3-(methyl-*N*-ethylcarbamate)-benz[f]indazol-4,9-dione (17)

N-Nitrosotetrahydro-2-pyrimidone (**9**, 0.6974 g, 5.4012 mmol) was added in small portions to a cooled mixture of KOH (0.9962 g) in methanol (10 mL) and diethyl ether (50 mL). The resultant mixture was stirred at room temperature for 25 min and washed with saturated NaCl (2 × 25 mL), 50% aqueous NaCl (2 × 25 mL), and saturated NaCl (2 × 25 mL). The ether layer was added to a solution of 1,4-naphthoquinone (0.4852 g, 3.0680 mmol) in THF (20 mL). The resultant mixture was stirred at room temperature for 1.5 h and filtered under suction to furnish a pale yellowish solid of **17** (444.8 mg, 48.44%). Mp 249–252 °C. IR (cm^{-1}) 3289, 3122, 1680, 1596. ^1H NMR (DMSO-d_6) δ 3.13 (t, 2H, J = 6.5 Hz), 3.51 (s, 3H), 7.25 (br t, 1H), 7.84–7.91 (m, 2H), 8.12–8.16 (m, 2H), 14.40 (s, 1H). ESI MS m/z 322.1 ([M+Na]$^+$ calcd 322.1).

4.4.1. 3-(Methyl-*N*-ethylcarbamate)-6-methylindazol-4,7-dione (18)

Mp 204–206 °C. IR (cm^{-1}) 3305, 3183, 3117, 1685, 1642, 1560. ^1H NMR (DMSO-d_6) δ 2.02 (d, 3H, J = 1.5 Hz), 3.00 (t, 2H, J = 6.9 Hz), 3.50 (s, 3H), 6.66 (d, 1H, J = 1.6 Hz), 7.25 (t, 1H, J = 5.7 Hz), 14.00 (s, 1H). ESI MS m/z 286.1 ([M+Na]$^+$ calcd 286.1).

4.4.2. 3-(Methyl-*N*-ethylcarbamate)-6-chloroindazol-4,7-dione (19)

Mp 192–193 °C. IR (cm^{-1}) 3322, 3179, 3109, 1700, 1642, 1572. ^1H NMR (DMSO-d_6) δ 3.03 (t, 2H, J = 6.8 Hz), 3.51 (s, 3H), 7.18–7.26 (m, 2H), 14.35 (s, 1H). EI HRMS calcd for $C_{11}H_{10}N_3O_4Cl$ m/z 283.0360, found 283.0320.

4.4.3. 3-(Benzyl-*N*-ethylcarbamate)-6-methoxylindazol-4,7-dione(20)

See Ref. 24.

4.4.4. 3-(Methyl-*N*-ethylcarbamate)-6-methoxylindazol-4,7-dione (21)

See Ref. 24.

4.4.5. 3-(Methyl-*N*-ethylcarbamate)-6,7-dichloro-5,8-dimethoxybenz[*f*]indazol-4,9-dione (22)

Mp 216–219 °C. IR (cm^{-1}) 3357, 3117, 1676, 1534. ^1H NMR (DMSO-d_6) δ 3.10 (br s, 2H), 3.51 (s, 3H), 3.86 (s, 3H), 3.87 (s, 3H), 7.25 (br s, 1H), 14.30 (s, 1H). ESI MS m/z 450.2 ([M+Na]$^+$ calcd 450.0).

4.4.6. 3-(Methyl-*N*-ethylcarbamate)-5-hydroxybenz[*f*]indazol-4,9-dione (23)

Mp 205–207 °C. IR (cm^{-1}) 3342, 3251, 1662, 1646, 1628, 1600. ^1H NMR (DMSO) δ 3.14 (br s, 2H), 3.51 (s, 3H), 7.14–7.44 (m, 2H), 7.58–7.87 (m, 2H), 12.60 (d, 1H), 14.50 (s, 1H). ESI MS m/z 338.1 ([M+Na]$^+$ calcd 338.1).

4.5. Expression and purification of recombinant human carbonyl reductase

Recombinant human carbonyl reductase was expressed in *Escherichia coli*, BL21 DE3 cells harboring *pET-5a-HCBR*.[26] The cells were grown in 6 L of LB-broth according to published methods.[26] Recombinant human liver carbonyl reductase was purified to homogeneity following a previously described method.[26] Throughout the preparation, human carbonyl reductase was distinguished from the bacterial enzyme by sensitivity to rutin inhibition.[28] Purity was confirmed via SDS–PAGE and the resulting protein was found to have k_{cat} and K_m values for menadione and 4-benzoylpyridine consistent with previously reported values for human carbonyl reductase.[21,29] Enzyme concentration was determined spectrophotometrically (ε_{280} = 0.699 $mg^{-1}ml$) and protein concentrations were determined by the method of Bradford using bovine serum albumin as the standard.[30]

4.6. Steady-state kinetics of carbonyl reductase

Kinetic measurements were made for the indazole-dione substrates with different substrate concentrations and 50 μM NADPH in 100 mM potassium phosphate. It was necessary to use up to 1% DMSO or 2% methanol for several of the carbonyl compounds to enhance their solubility. In control experiments using the standard assay described below, the enzyme was unaffected by methanol concentrations up to 2% and DMSO concentrations up to 1%. Carbonyl substrate concentrations were determined from mass or spectrophotometrically (menadione, ε_{340} = 2500 M^{-1} cm^{-1}; daunorubicin, ε_{486} = 12,100 M^{-1} cm^{-1}). Assays were measured by following NADPH oxidation to $NADP^+$ spectrophotometrically (ε_{340} = 6220 M^{-1} cm^{-1}) as a function of time using a Varian Cary 100 Bio.[31] Initial slopes of the resulting progress curves were estimated from fits of the curves to either a line or a parabola, and these slopes were used to calculate the initial velocities. Steady-state kinetic data were fit to the Michaelis–Menten equation using HYPERW.[32]

4.7. Inhibition of carbonyl reductase

Inhibitors were evaluated by determining the IC_{50} values in the following standard assay. Enzyme was added to different concentrations of inhibitor, 250 μM menadione, and 50 μM NADPH in 100 mM potassium phosphate, pH 7.0 at 25 °C. IC_{50} values were extrapolated from the plot of % activity remaining versus inhibitor concentration. For more complete inhibition studies, concentrations of indazole-diones **11** and **12** were varied against varied menadione or NADPH concentrations. For experiments with varied menadione concentrations the NADPH concentrations were fixed at either 10 μM, 50 μM, or 300 μM. For experiments with varied NADPH concentrations, the menadione concentrations were held constant at either 50 μM or 250 μM. The resulting data were fit to competitive, uncompetitive, and noncompetitive inhibition equations using COMP, UNCOMP, and NONCOMP, respectively.[32]

4.8. Computational chemistry for select indazole-diones

Indazole-diones **11**, **12**, **15**, **17**, **22**, and **23** were subjected to a conformation search using Hartree–Fock 3-21G* with SPARTAN 04.[33] The two lowest energy conformers for each compound were then geometry optimized in SPARTAN 04 using Hartree–Fock 6-31G*. The remaining structures were subjected to a conformer search excluding the carbamate side-chain using Hartree–Fock 3-21G* with SPARTAN 04. For each indazole-dione, the lowest energy conformers within a calculated 2 kcal/mol range of each other were carried forward by appending the carbamate side-chain from the initial studies onto the quinone containing core. Another geometry optimization was done with the side-chain intact using Hartree–Fock 3-21G*. These structures were then imported into GAUSSIAN 03[34] and subjected to a geometry optimization and frequency calculation using Hartree–Fock 6-31G*. For each indazole-dione, the lowest energy structures were analyzed for conformational and electronic differences and similarities.

Acknowledgments

The work described in this paper was supported in part by NIH NCRR P20-RR16454 (H.A.C. and A.S.), NIH NCI 1 R15 CA102119-01 (H.A.C. and C.L.), Research Corporation Cottrell College Science Award CC5404 (H.A.C.), Howard University New Faculty Grant (O.B.) and Grant 2 G12 RR003048 from the RCMI Program, Division of Research Infrastructure, National Center for Research Resources, NIH.

References and notes

1. Young, R. C.; Ozols, R. F.; Myers, C. E. *N. Eng. J. Med.* **1981**, *305*, 139–153.
2. Cortes, E. P.; Lutman, G.; Wanka, J.; Wang, J. J.; Pickren, J.; Wallace, J.; Holland, J. F. *Cancer Chemother. Rep.* **1975**, *6*, 215–255.
3. Minow, R. B. R.; Gottleeb, J. *Cancer Chemother. Rep.* **1975**, *6*, 195–210.
4. von Hoff, D. D.; Layard, M. W.; Basa, P.; Davis, H. L., Jr.; von Hoff, A. L.; Rozencweig, M.; Muggia, F. M. *Ann. Int. Med.* **1979**, *91*, 710–717.
5. Lipshultz, S. E.; Colan, S. D.; Gelber, R. D.; Perez-Atyde, A. R.; Sallan, S. E.; Sanders, S. P. *N. Eng. J. Med.* **1991**, *324*, 808–815.
6. Horenstein, M. S.; Vander Heide, R. S.; L'Ecuyer, T. J. *Mol. Genet. Metab.* **2000**, *71*, 436–444.
7. Schimmel, K. J. M.; Richel, D. J.; van den Brink, R. B. A.; Guchelaar, H.-J. *Cancer Treat. Rev.* **2004**, *30*, 181–191.
8. Wojtacki, J.; Lewicka-Nowak, E.; Lesniewski-Kmak, K. *Med. Sci. Monit.* **2000**, *6*, 411–420.
9. Cusack, B. J.; Gambliel, H.; Musser, B.; Hadjokas, N.; Shadle, S. E.; Charlier, H.; Olson, R. D. *Cancer Chemother. Pharmacol.* **2006**, *58*, 517–526.
10. Olson, R. D.; Mushlin, P. S.. *Mechanism of Anthracycline Cardiotoxicity: Are Metabolites Involved?* In *Cellular and Molecular Toxicology in In Vitro Toxicology*; CRC Press: Boca Raton, 1990.
11. Olson, R. D.; Mushlin, P. S.; Brenner, D. E.; Fleischer, S.; Cusack, B. J.; Chang, B. K., ; Robert, R. J., Jr. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 3585–3589.
12. Boucek, R. J., Jr.; Olson, R. D.; Brenner, D. E.; Ogunbunmi, E. M.; Inui, M.; Fleischer, S. *J. Biol. Chem.* **1987**, *262*, 15851–15856.
13. Cusack, B. J.; Mushlin, P. S.; Voulelis, L. D.; Li, X.; Boucek, R. J., Jr.; Olson, R. D. *Toxicol. Appl. Pharmocol.* **1993**, *118*, 177–185.
14. Minotti, G.; Cavaliere, A. F.; Mordente, A.; Rossi, M.; Schiavello, R.; Zamparelli, R.; Possati, G. *J. Clin. Invest.* **1995**, *95*, 1595–1605.
15. Minotti, G.; Recalcati, S.; Mordente, A.; Liberi, G.; Calafiore, A. M.; Mancuso, C.; Preziosi, P.; Cairo, G. *FASEB J.* **1998**, *12*, 541–551.
16. Minotti, G.; Cairo, G.; Monti, E. *FASEB J.* **1999**, *13*, 199–211.
17. Olson, R. D.; Li, X.; Palade, P.; Shadle, S. E.; Mushlin, P. S.; Gambliel, H. A.; Fill, M., ; Boucek, R. J., Jr.; Cusack, B. J. *Toxicol. Appl. Pharmacol.* **2000**, *169*, 168–176.
18. Forrest, G. L.; Gonzalez, B.; Tseng, W.; Li, X.; Mann, J. *Cancer Res.* **2000**, *60*, 5158–5164.
19. Olson, L. E.; Bedja, D.; Alvey, S. J.; Cardounel, A. J.; Gabrielson, K. L.; Reeves, R. H. *Cancer Res.* **2003**, *63*, 6602–6606.
20. Ax, W.; Soldan, M.; Koch, L.; Maser, E. *Biochem. Pharmacol.* **2000**, *59*, 293–300.
21. Wermuth, B. *J. Biol. Chem.* **1981**, *256*, 1206–1213.
22. Iwata, N.; Inazu, N.; Satoh, T. *J. Biochem.* **1989**, *105*, 556–564.
23. Tanaka, M.; Bateman, R.; Rauh, D.; Vaisberg, E.; Ramachandani, S.; Zhang, C.; Hansen, K. C.; Burlingame, A. L.; Trautman, J. K.; Shokat, K. M.; Adams, C. L. *PLoS Biol.* **2005**, *3*, 764–776.
24. Bakare, O.; Zalkow, L. H.; Burgess, E. M. *Synth. Commun.* **1997**, *27*, 1569–1576.
25. Barakat, M. Z.; Shehab, S. K.; El-Sadr, M. M. *J. Chem. Soc.* **1958**, 901–902.
26. Slupe, A.; Williams, B.; Larson, C.; Primbs, T.; Lee, L. M.; Breusch, A. J.; Bjorklund, C.; Warner, D. L.; Peloquin, J.; Shadle, S. E.; Gambliel, H. A.; Cusack, B. J.; Olson, R. D.; Charlier, H. A., Jr. *Cardiovasc. Toxicol.* **2005**, *5*, 365–376.
27. Tanaka, M.; Zhang, C.; Shokat, K. M.; Burlingame, A. L.; Hansen, K.; Bateman, R. L.; Dimagno, S. G. Pyrazolo Pyrimidine Derivatives and Methods of Use Thereof. In *WHO*: PCT, 2005.
28. Bohren, K. M.; Wermuth, B.; Harrison, D.; Dagmar, R.; Petsko, G. A.; Gabbay, K. H. *J. Mol. Biol.* **1994**, *244*, 659–664.
29. Bohren, K. M.; von Wartburg, J. P.; Wermuth, B. *Biochem. J.* **1987**, *244*, 165–171.
30. Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248–254.
31. Wermuth, B.. *Aldo–Keto Reductases*. In *Enzymology of Carbonyl Metabolism: Aldehyde Dehydrogenase, Aldo/Keto Reductase, and Alcohol Dehydrogenase*; Alan R. Liss: New York, 1985.
32. Cleland, W. W. *Methods Enzymol.* **1979**, *63*, 103–138.

33. SPARTAN '04 *Windows*; Version 1.0.1 ed.; Wavefunction: Irvine, CA, (2004).
34. Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Montgomery, Jr., J. A.; Vreven, T.; Kudin, K. N.; Burant, J. C.; Millam, J. M.; Iyengar, S. S.; Tomasi, J.; Barone, V.; Mennucci, B.; Cossi, M.; Scalmani, G.; Rega, N.; Petersson, G. A.; Nakatsuji, H.; Hada, M.; Ehara, M.; Toyota, K.; Fukuda, R.; Hasegawa, J.; Ishida, M.; Nakajima, T.; Honda, Y.; Kitao, O.; Nakai, H.; Klene, M.; Li, X.; Knox, J. E.; Hratchian, H. P.; Cross, J. B.; Bakken, V.; Adamo, C.; Jaramillo, J.; Gomperts, R.; Stratmann, R. E.; Yazyev, O.; Austin, A. J.; Cammi, R.; Pomelli, C.; Ochterski, J. W.; Ayala, P. Y.; Morokuma, K.; Voth, G. A.; Salvador, P.; Dannenberg, J. J.; Zakrzewski, V. G.; Dapprich, S.; Daniels, A. D.; Strain, M. C.; Farkas, O.; Malick, D. K.; Rabuck, A. D.; Raghavachari, K.; Foresman, J. B.; Ortiz, J. V.; Cui, Q.; Baboul, A. G.; Clifford, S.; Cioslowski, J.; Stefanov, B. B.; Liu, G.; Liashenko, A.; Piskorz, P.; Komaromi, I.; Martin, R. L.; Fox, D. J.; Keith, T.; Al-Laham, M. A.; Peng, C. Y.; Nanayakkara, A.; Challacombe, M.; Gill, P. M. W.; Johnson, B.; Chen, W.; Wong, M. W.; Gonzalez, C.; Pople, J. A. GAUSSIAN *03, Revision C.02*; Gaussian: Wallingford, CT, 2004.