Acetylation of 1,2,5,8-tetrahydroxy-9,10-anthraquinone Improves Binding to DNA and Shows Enhanced Superoxide Formation that Explains Better Cytotoxicity on *JURKAT T Lymphocyte Cells*

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Abstract: *Background*: Hydroxy-9,10-anthraquinones form the core unit of anthracycline anticancer drugs and are close structural analogues to these drugs. Although they show close resemblance to anthracyclines in physicochemical characteristics and electrochemical behavior their biophysical interactions are somewhat weaker than anthracyclines which is a disadvantage. One reason is the formation of anionic species by hydroxy-9,10-anthraquinones. Hence if formation of anionic species is prevented there could be a possibility hydroxy-9,10-anthraquinones would bind DNA better.

Procedure: For this 1, 2, 5, 8-tetrahydroxy-9,10-anthraquinone (THAQ) was acetylated to obtain a tetra-acetylated derivative (THAQ-ace) whose interaction with calf thymus DNA was studied using UV-Vis spectroscopy at different pH.

Results: Binding constant values for THAQ-ace (~10^5) were higher than THAQ at different pH. Increase in binding constant was attributed to anionic species not formed for THAQ-ace at physiological pH. Hence, unlike THAQ, binding constant values for THAQ-ace interacting with calf thymus DNA did not show variation with pH. In fact, it remained more or less constant. Increase in size of the acetylated form (THAQ-ace) compared to THAQ had a negative influence on binding. THAQ-ace showed enhanced superoxide formation. Both DNA binding and superoxide formation were responsible for a significant improvement in anticancer activity for THAQ-ace compared to THAQ on *Jurkat T lymphocyte cells*.

Conclusion: Binding constant values for THAQ-ace binding to DNA were close to that reported for some standard anthracyclines. Hence, suitable modification of the less costly hydroxy-9,10-anthraquinones could provide alternatives to anthracyclines in cancer chemotherapy.

Keywords: Acetylated 1,2,5,8-tetrahydroxy-9,10-anthraquinone (THAQ-ace), anthracycline, calf thymus DNA, superoxide, *JURKAT T lymphocyte cells*.

INTRODUCTION

In an effort aimed at creating new antineoplastic drugs, hydroxy-9,10-anthraquinones could be chosen as substitutes of anthracyclines owing to close structural similarity and high affinity towards DNA [1-8]. However, hydroxy-9,10-anthraquinones under physiological conditions generate anions that tend to reduce their ability to interact with DNA [1]. A substitution at the 2-hydroxy position of 1,2,5,8-tetrahydroxy-9,10-anthraquinone could prevent dissociation of the –OH group which in turn could prevent formation of anionic species. When this is done it should help to increase binding constants of THAQ with DNA. On the other hand, hydroxy-9,10-anthraquinones are enzymatically reduced to a semiquinone radical, able to transfer an electron to molecular oxygen forming superoxide radical anion, O_2^- which is eventually converted to hydrogen peroxide by dismutation [4, 9]. Hydrogen peroxide generates hydroxyl radicals responsible for DNA damage [10, 11]. In a recent study, we demonstrated as pH increases, there was a decrease in the binding of THAQ with calf thymus DNA indicating negatively charged species prevented proper interaction [1]. This work was done with a view to find out if by acetylating the 2-hydroxy position of 1,2,5,8-tetrahydroxy-9,10-anthraquinone (THAQ) that is responsible for generation of the anion, the new molecule was able to bind calf thymus DNA better [1].

THAQ and other hydroxy-9,10-anthraquinones are structural analogues of anthracyclines and are less costly [1-8]; more specifically THAQ resembles carminomycin and nogalamycin [1, 12-14]. In an effort to see if interaction of THAQ with c t DNA at physiological pH was close to that reported for these drugs we concluded dissociation of the proton from the phenolic–OH at C_2 was a hindrance to effective binding [1]. Since THAQ exists in two forms at physiological pH (neutral and anionic), overall binding was a consequence of both [1, 2]. While contribution of the neutral form of THAQ towards intrinsic binding with c t

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DNA was high, a comparatively lower contribution from the anionic form resulted in a low overall binding constant [1]. Thus, formation of anionic species had to be prevented and a way to do it was to substitute the phenolic –OH proton at C_2. Results indicated if this could be done then the overall binding constant for THAQ would increase considerably [1]. Hence, an attempt was made to prepare a 2-acetyl derivative of THAQ using acetic anhydride following standard procedure [15]. However, our efforts to acetylate only the phenolic–OH at C_2 failed and every time a mixture of products was obtained. It was then that we decided to acetylate THAQ exhaustively so that a tetra-acetylated derivative (THAQ-ace) was formed as the sole product. Previous reports on hydroxy-9,10-anthraquinones showed although binding constants were high, they were lower than anthracyclines [1-8, 11-13]. Unlike the anthracyclines, THAQ has no sugar units in its structure which was a disadvantage in comparison to the established drugs [13, 14, 16]. However, the advantage with hydroxy-9, 10-anthraquinones is that they are readily available and less costly than anthracyclines. Cost being important in drug design, we tried to see if a modified THAQ in the form THAQ-ace was able to match the biophysical parameters reported for anthracyclines [1-8].

METHODS AND MATERIALS

Preparation of THAQ-ace

0.02 gram of THAQ was dissolved in 5 ml 3 M NaOH and poured in 10-20 gram crushed ice taken in a beaker followed by the addition of 1 ml acetic anhydride with vigorous shaking [15]. Treatment of dilute HCl formed a reddish brown mass. The product was recovered and re-crystallized from ethanol-water mixtures. It was characterized by ^{1}H NMR in $CDCl_3$ using tetramethylsilane (TMS) as internal standard and by mass spectroscopy. Product Yield: 75%. Anal. Calc. (%) for $C_{22}H_{16}O_{10}$: C:60.00 H:3.64; Found: C: 59.73; H:3.69. We would like to mention here that THAQ-ace that we prepared for reasons mentioned above is not an altogether new compound. It is already reported in the literature and has a CAS 2885-46-3. However, we prepared it for our study.

^{1}H NMR (Figure **1**) clearly indicates peaks due to phenolic –OH of THAQ were absent while those due to protons of the methyl groups of acetyl units were present. The mass spectra (Figure **2**) indicate all phenolic –OH groups in THAQ were acetylated.

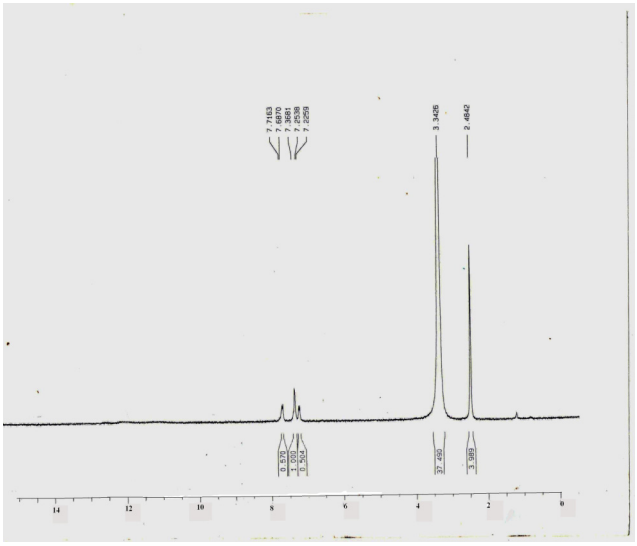


Figure 1: ^{1}H NMR of THAQ-ace.

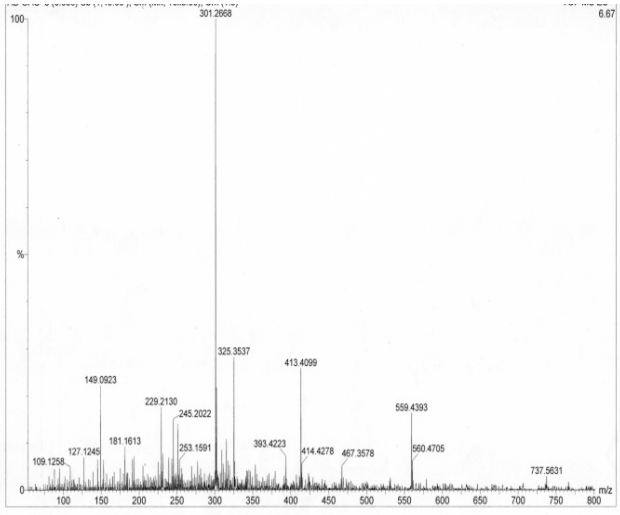
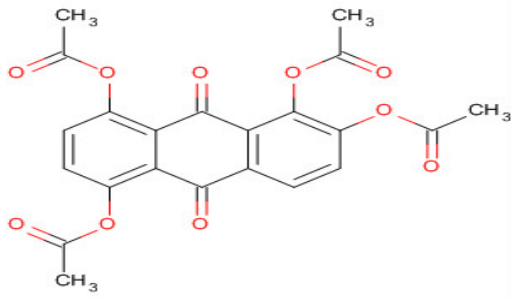


Figure 2: Mass Spectra of THAQ-ace.

The molecular ion peak was found at m/z = 559.43 and 560.47. The peak was attributed to THAQ-ace bound to three molecules of acetonitrile, the solvent used for the study. The peak at m/z = 467.35 was that of THAQ-ace bound to Na and 4 H. From m/z = 467.35 dissociation of two acetyl groups and Na should yield a fragment with m/z = 326.35. The mass spectrum shows a peak at m/z = 325.35 that explains this species. Peaks at m/z = 413.40 & 414.42 (m/z_{theo} = 413.36) were obtained following dissociation of two methyl groups from THAQ-ace. An experimental peak at m/z = 393.42 was due to the fragment generated by loss of one acetyl unit and four H atoms from THAQ-ace (m/z_{theo} = 393.36). The experimental peak at m/z = 301.26 was obtained due to three different species originating from independent dissociations of THAQ-ace.



THAQ-ace

DNA Preparation

Calf thymus DNA was purchased from Sisco Research Laboratories, India and dissolved in phosphate buffer for approximately 5 h. A molar extinction coefficient of 6600 $M^{-1}cm^{-1}$ (base) at 260 nm was used to calculate its concentration. Absorbance at 280 nm was also recorded. A_{260}/A_{280} was obtained in the range 1.8 to 1.9 indicating the DNA was sufficiently free of protein. Quality of c t DNA was also checked by the characteristic CD band at 260 nm.

Interaction of the Compounds with DNA

Keeping concentration of THAQ-ace constant at 45 μM, c t DNA was gradually added. Addition was continued till saturation was reached. Using different equations, interaction of THAQ-ace with c t DNA was examined at different pH.

$$L + DNA \rightleftharpoons L - DNA \qquad K_d = \frac{[L][DNA]}{[L - DNA]} \tag{1}$$

L represents THAQ-ace while K_d the dissociation constant [1, 4-8]. Eq. 1 yields Eq. 2 where reciprocal of the change in absorbance was plotted against reciprocal of (C_D - C_0). C_D and C_0 were concentrations of c t DNA and THAQ-ace respectively. Eq. 2 yields ΔA_{max}, the maximum change in absorbance at the pH at which titration was performed and K_{app} (= $1/K_d$).

$$\frac{1}{\Delta A} = \frac{1}{\Delta A_{max}} + \frac{K_d}{\Delta A_{max}(C_D - C_0)} \tag{2}$$

$$K_d = \frac{\left[C_{0-}\left(\frac{\Delta A}{\Delta A_{max}}\right)C_0\right]\left[C_D - \left(\frac{\Delta A}{\Delta A_{max}}\right)C_0\right]}{\left(\frac{\Delta A}{\Delta A_{max}}\right)C_0} \tag{3}$$

$$C_0\left(\frac{\Delta A}{\Delta A_{max}}\right)^2 - (C_0 + C_D + K_d)\left(\frac{\Delta A}{\Delta A_{max}}\right) + C_D = 0 \tag{4}$$

The titrimetric data was fitted to another form of a double reciprocal plot [Eq. 5] and binding constant was evaluated [2].

$$\frac{1}{(C_b / C_0)} = 1 + \frac{1}{[K][C_D]}. \tag{5}$$

Intrinsic binding constant K^* and binding stoichiometry "n" in terms of bound molecules per nucleic acid base was determined according to Eq. 6 [17].

$$r/C_f = K'(n - r) \tag{6}$$

NADH Dehydrogenese Assay

An enzyme assay was performed at 25°C with cytochrome c as the electron acceptor [18]. Both THAQ and THAQ-ace were used in the assay for NADH-cytochrome c reductase activity where the reduction of cytochrome c at 550 nm was followed at pH ~ 7.4 (0.05 M phosphate buffer). Test solutions contained 80.0 μM cytochrome c, 160.0 μM NADH, 3 UI^{-1} NADH dehydrogenase and compounds (THAQ/THAQ-ace) in the concentration range 0 to 60.0 μM. Activity of NADH dehydrogenase has been expressed in units where one unit of activity reduces 1.0 μ mole oxidized cytochrome c per minute at pH 7.4 at 25°C. Formation of superoxide radical anion catalyzed by THAQ or THAQ-ace was measured from the reduction of cytochrome c inhibited by SOD in the presence of NADH and NADH dehydrogenase [3, 19-21]. Enzyme assay was done using the kinetics software of JASCO–V630, Japan.

Studies on JURKAT Cells

Cells were seeded at 2 x 10^4 cells/well in a 96-well plate with different concentrations (0 - 100 μM) of THAQ/THAQ-ace and observed for 24 hours by the MTT assay [22]. Briefly, cells were incubated in triplicate having different concentrations of THAQ and THAQ-ace at 37°C. Three hours before completion of time points, MTT solution (5 mg/mL) was added to each well. Formazone crystals were formed at the end of three hours, representing percentage cell viability assessed at 560 nm. Cell apoptosis (100%) was obtained by lysis of cells in 5% SDS buffer. Percentage cell viability was calculated as below [22].

% cell viability = 100 x ($O.D._{.sample}$ – $O.D._{.100\ \%\ lysis}$) / ($O.D._{.0\%\ lysis}$ – $O.D._{.100\ \%\ lysis}$)

RESULTS AND DISCUSSION

We have been making an effort to show hydroxy-9,10-anthraquinones resemble anthracyclines in structure and function [1, 3-8]. The ability of anthracyclines to form semiquinones and reactive oxygen species is important for cytotoxicity [23]. Earlier it was shown 1,2-dihydroxy-9,10-anthraquinone forms semiquinone leading to the generation of superoxide [3]. A lot of emphasis is laid on the ability of hydroxy-9,10-anthraquinones to bind DNA so that their efficacy may be compared with anthracyclines with regard to DNA binding, topoisomerase inhibition and radical induced DNA damage [10, 11, 24-26]. Moreover, drug-DNA interactions are key issues in stopping DNA replication or RNA transcription in cancer cells [23-25]. Hence a low binding constant recorded earlier for THAQ with DNA is certainly not desirable [1]. For this reason, we modified THAQ to the acetylated THAQ-ace to see if the latter was better in binding DNA.

Figure **3** shows a gradual decrease in absorbance of THAQ-ace when titrated with c t DNA at pH 7.6 and 8.2 respectively. It is worth mentioning here that the titration of THAQ-ace with c t DNA did not show significant changes in λ_{max} with pH unlike that observed earlier for THAQ [1]. λ_{max} remained almost constant (552-556 nm). Change in absorbance of THAQ-ace at 554 ± 2 nm, at different pH was used to construct binding isotherms that were analyzed by non-linear

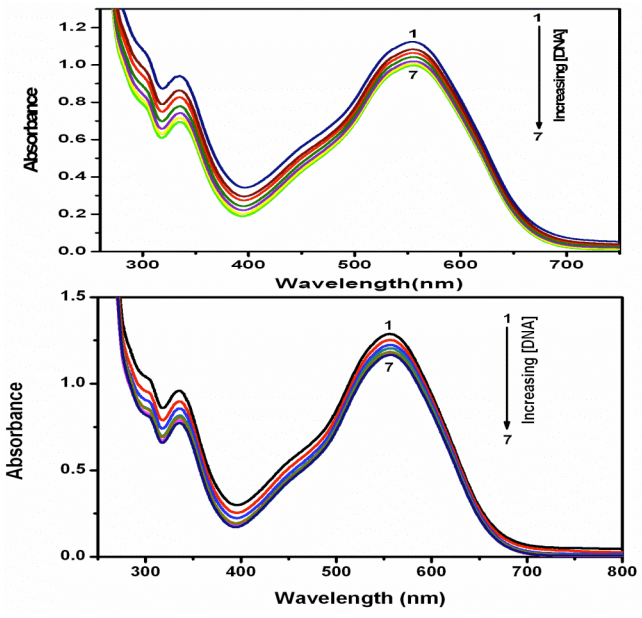


Figure 3: Absorption spectra of THAQ-ace (45μM) with c t DNA in phosphate buffer containing 120mM NaCl, 3.5mM KCl, 1mM $CaCl_2$ & 0.5mM $MgCl_2$ at pH 7.6 (above) & 8.2 (below); (1) 0 μM, (2) 21.0 μM, (3) 62.9 μM, (4) 104.7 μM, (5) 208.4 μM, (6) 412.6 μM, (7) 711.6 μM ct DNA. Temp.=303 K.

curve fit [Eq. 4] and double-reciprocal plots [Eqs. 2 and 5]. These were based on the assumption absorbance was linearly proportional to concentration of THAQ-ace [1, 4-8].

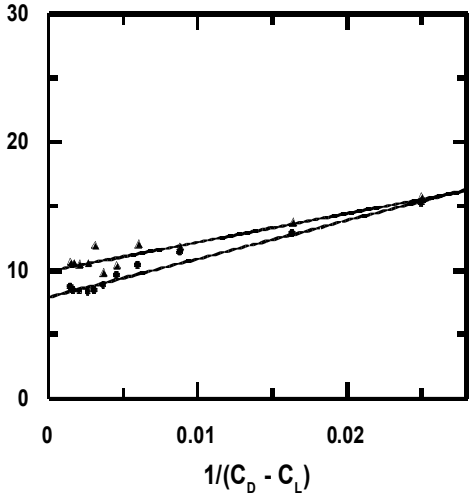


Figure 4: Double reciprocal plots for reciprocal of ΔA against reciprocal of ($C_D - C_L$). [THAQ-ace]= 45μM, [NaCl]= 120 mM, [KCl] = 3.5 mM, [$CaCl_2$] = 1 mM, [$MgCl_2$] = 0.5 mM; pH: 7.6 (•), 8.2 (▲) Temp. = 303 K.

Decrease in absorbance at 552 nm (pH 6.8-7.6), 554 nm (pH 7.8-8.0) and 556 nm (pH 8.2) upon adding c t DNA enabled determination of ΔA. Figure **4** shows double reciprocal plots while Figure **5** & **1S** show non-linear plots for the titration of THAQ-ace with c t DNA at pH 7.6 and 8.2 respectively. It appears from Figure **4** that lines are very close to each other with almost no difference for change in pH, unlike that recorded earlier for THAQ [1]. Plot of $\Delta A/\Delta A_{max}$ against [DNA]/[THAQ-ace] at pH 7.6 [Inset of Figure **5**] and pH 8.2 [Inset of Figure **1S**] yielded n_b the site size as 2.0.

Multiplying K_{app} with "n_b," K^*, the intrinsic binding constant was determined at each pH (Table **1**) [27]. Apparent and intrinsic binding constants were also evaluated using Eq. 5 (Figure **2S**).

Intrinsic binding constant and site size of interaction at different pH was also evaluated according to Scatchard (Table **1**) [17, 27]. Figure **6** is a typical plot obtained at pH 7.6. Values for K_{app} and K^* determined in this study were not only higher than THAQ at each pH but they were constant at all pH contrary to results obtained earlier for THAQ [1]. Values for n_b from the plots of $\Delta A/\Delta A_{max}$ against [DNA]/[THAQ-ace] at each pH were in excellent agreement with the Scatchard plots (Table **1**). This was expected if dissociation of the proton on the phenolic –OH at C_2 of THAQ could be prevented [1].

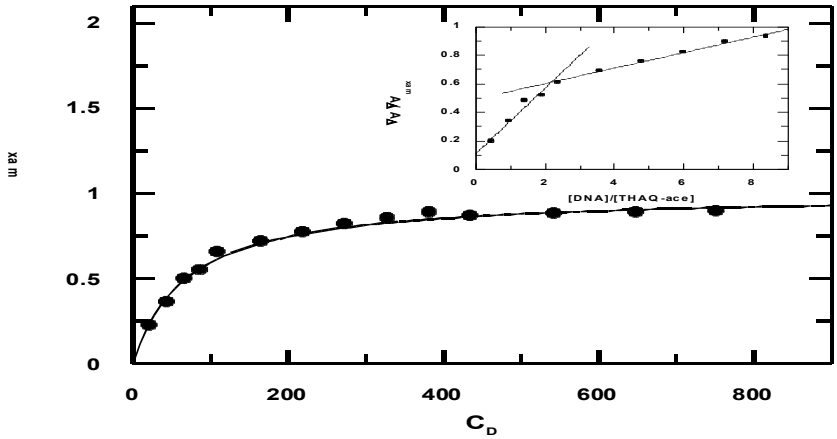


Figure 5: Binding isotherm for interaction of THAQ-ace with c t DNA. Dark line shows the fitted data [Eq (4)]. [THAQ]= 45μM, [NaCl]= 120 mM, [KCl]= 3.5 mM, [$CaCl_2$]= 1 mM, [$MgCl_2$]= 0.5 mM; pH= 7.6; Temp.= 303 K. **Inset:** Plot of normalized increase of absorbance as a function of mole-ratio of c t DNA to THAQ.

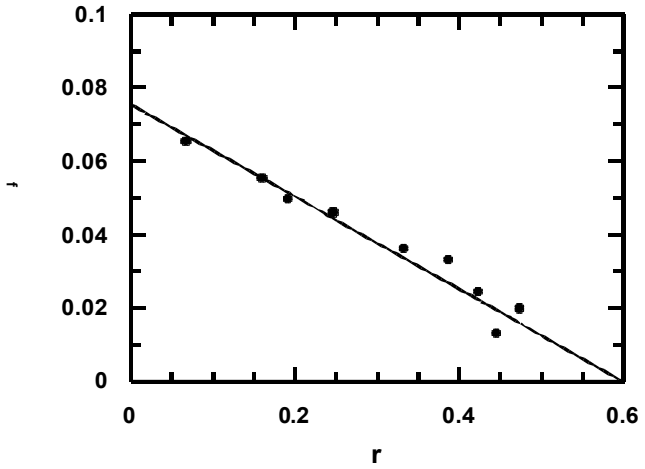


Figure 6: Scatchard plot for the interaction of THAQ-ace with ct DNA. [THAQ] = 45μM, [NaCl] = 120 mM, [KCl] = 3.5 mM, [$CaCl_2$] = 1 mM, [$MgCl_2$] = 0.5 mM; pH = 7.6; Temp. = 303K.

Considering the contributions of the neutral and anionic forms of THAQ to the overall binding constants we suggested earlier if THAQ were to exist only in the neutral form then the intrinsic binding constant with c t DNA would increase substantially [1]. Prediction for K^* of THAQ binding with c t DNA was in the range 5.46 × 10^5 M^{-1} to 7.53 × 10^5 M^{-1} [1]. However, values obtained in this study for intrinsic binding constant of THAQ-ace, having no anionic form at physiological pH was in the range 0.83 × 10^5 M^{-1} to 1.26 × 10^5 M^{-1}. As can be seen the values are slightly lower than our prediction [1]. It would be important to keep in mind the fact that in acetylating all four phenolic –OH groups of THAQ, the molecular weight of the compound (THAQ-ace) increased from 272 (for THAQ) to 440 (for THAQ-ace) (an increase by ~1.6 times). Hence, one reason why experimental values for intrinsic binding constants for THAQ-ace did not match our earlier predictions could be steric in origin. Had we been able to acetylate only the phenolic –OH at C_2 the molecular weight would have increased only marginally (~1.15 times) and experimental values could have been closer to our predictions [1]. Although binding constant values for the tetra-acetylated derivative (THAQ-ace) did not increase to our expectations, yet it did show substantial increase that helped to reduce the difference between hydroxy-9,10-anthraquinones and anthracyclines binding c t DNA [1, 4-8].

Acetylation of THAQ proved a few points in our approach to modifying hydroxy-9,10-anthraquinones for better binding with c t DNA. Owing to acetylation, no anionic species formed at physiological pH that helped to increase intrinsic binding constants. Although an increase in size for THAQ-ace (compared to THAQ) could have been a hindrance to better interaction with DNA, presence of acetyl units might have had some positive role, since it enabled THAQ-ace to be more effective on *JURKAT T lymphocyte cells* (discussed later). These interesting observations pertaining to acetylation of THAQ support the role of sugar units in anthracyclines and proclaim that suitably modified hydroxy-9,10-anthraquinones (like THAQ-ace) could be considered as alternatives to anthracyclines [16, 28].

Figure **7** relates the ratio of input compound/DNA (r_i) to the ratio of bound compound/DNA (r_b) as derived from Scatchard calculations [29]. In this plot, a 45^0 line indicates that the entire compound present was bound to DNA [29]. The curves obtained for THAQ-ace at pH 7.6, 7.8 and 8.2 were close to each other like that observed for other plots indicating similarity in binding at different pH.

Table 1: A Comparison of Binding Constant Values for the Interaction of THAQ-ace with c t DNA at Different pH

pH	Apparent binding constants $K_{app}\times10^{-4}$ (M^{-1})		Site size (n_b)	Overall binding constant $K'\times10^{-4}$ M^{-1}) [$K^* = K_{app} \times n_b$]	Overall binding constant $K^*\times10^{-4}$ (M^{-1}) (Scatchard plot)	Site size (n_b) Scatchard plot	Overall binding constant from double reciprocal plot with intercept = 1 $K^*\times10^{-4}$ (M^{-1})
	From double reciprocal plot	From non linear–fit					
6.8	4.46	4.56	2.0	9.02	8.20	1.8	7.80
7.2	4.62	4.90	2.0	9.52	8.31	1.8	7.85
7.6	5.10	4.37	2.0	9.47	9.29	2.0	8.23
7.8	5.16	4.14	2.0	9.30	7.95	1.8	8.46
8.0	4.53	5.06	2.0	9.59	9.96	2.2	7.97
8.2	4.43	5.02	2.0	9.45	8.26	1.9	7.46

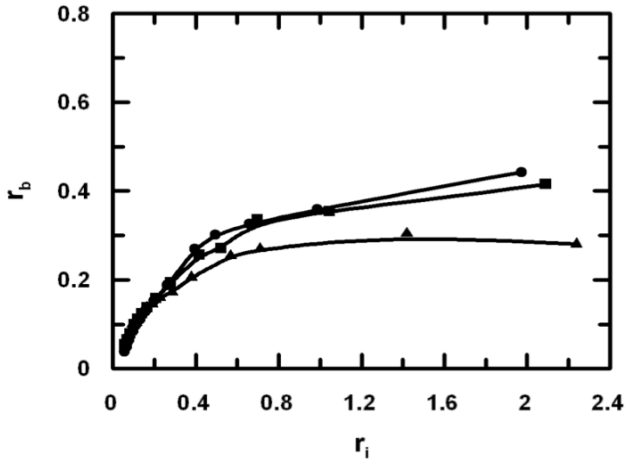


Figure 7: Relationship of input THAQ-ace/DNA ratio (r_i) to the bound THAQ-ace/DNA ratio (r_b) as calculated from decrease in absorbance following interaction of THAQ-ace with c t DNA at pH 7.6 (•), 7.8 (▲) and 8.2 (■) respectively having 120 mM NaCl, 3.5 mM KCl, 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$. Temp. = 303K.

Formation of superoxide radical anion catalyzed by THAQ and THAQ-ace was measured as described in Experimental. Results revealed superoxide formation was higher in case of THAQ-ace under similar experimental conditions (Figure **8**). Thus with all hydroxyl groups acetylated in THAQ-ace, stabilization of the semiquinone by hydrogen bonding probably decreased as a result of which there occurred increased conversion of semiquinone to superoxide. Semiquinones and superoxides being responsible for cytotoxic action could be one reason why besides an increased DNA binding, THAQ-ace was more effective on *JURKAT T lymphocyte cells* [10, 11, 23].

Anti-proliferative activity of THAQ-ace and THAQ on *JURKAT T lymphocyte cells* (Figure **9**) showed THAQ-ace was more effective suggesting acetylation of THAQ was an important manifestation that could find a clinical outcome. While results were almost similar for 10 µM, beyond 25 µM THAQ-ace was clearly able to decrease cell viability. While IC_{50} for THAQ-ace was ~60 µM that for THAQ was in the range 170-175 µM (not shown in Figure **9**).

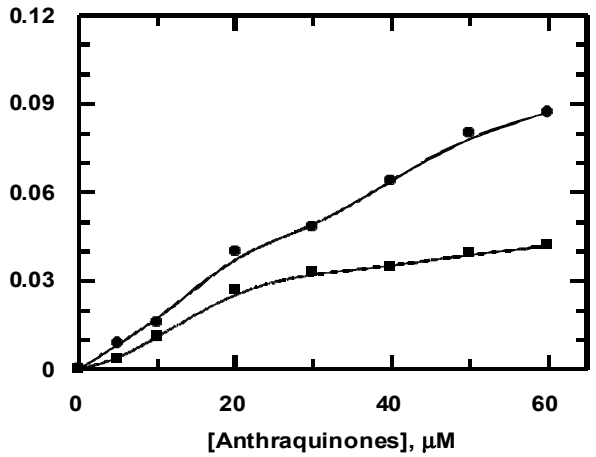


Figure 8: Effect of THAQ and THAQ-ace on superoxide formation by NADH dehydrogenase. Superoxide formation was determined spectrophotometrically by the rate of superoxide dismutase-inhibitable cytochrome-c reduction. The reaction mixture contained 100 mM Hepes buffer (pH 7.2), 80.0 µM cytochrome c, 160.0 µM NADH, 10 UI^{-1} NADH *dehydrogenase, 0 or 40.0 µg ml^{-1} SOD and the indicated amount of compound. [▪,THAQ; ●, THAQ-ace].

CONCLUSION

The tetra-acetylated derivative of THAQ on the one hand showed increased tendency to bind c t DNA while on the other generated more reactive oxygen species than THAQ. These two together made it a better anticancer agent on *JURKAT T lymphocyte cells*. Another advantage in favor of THAQ-ace was that the starting material (THAQ) is easily available and the

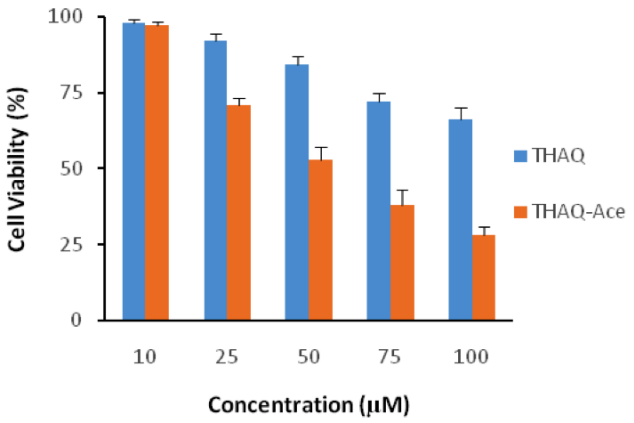


Figure 9: Effects of THAQ and THAQ-ace on *JURKAT T lymphocyte cell* viability. Plot represents a comparative profile of the effective cytotoxicity of the compounds at different concentrations (0 − 100) µM on *JURKAT T lymphocyte cells* after 24 hours of treatment. Each point depicts mean ± S.D. of three independent experiments.

conversion not difficult. The other important finding is that acetyl units present on THAQ-ace probably helped in cellular interactions as a result of which inspite of its large size, as compared to THAQ, THAQ-ace was more effective on *JURKAT T lymphocyte* cells. Hence, THAQ-ace considering its close similarity to anthracycline drugs, with further modifications could be developed as a less costly alternative.

ACKNOWLEDGMENT

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ABBREVIATIONS

THAQ = 1,2,5,8-tetrahydroxy-9,10-anthraquinone

c t = calf thymus

SUPPLEMENTAL MATERIALS

The supplemental materials can be downloaded from the journal website along with the article.

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