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FURTHER STUDIES ON THE INFLUENCE OF PERIPHERAL RING SUBSTITUTION ON THE CARCINOGENICITY OF TRICYCLOQUINAZOLINE

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Abstract—3-Ethyl-, 3-t-butyl-, 3-methoxy-, and 2-fluoro-tricycloquinazoline have been unequivocally synthesised. Determinations of their epidermal carcinogenic activities and further studies on 2-methyl-TCQ have been carried out.

The inactivity of 2-methyl-TCQ, both as a carcinogen and as an initiator, has been confirmed, whereas 2-fluoro-TCQ was found to be active in both respects. Substitution in the 2-position of TCQ is therefore not in itself sufficient to abolish activity, and, moreover, covalent bonding of the 2-position to a receptor is not involved in TCQ carcinogenesis. Results with 3-methoxy-TCQ indicated that this substituent does not have a specific structural effect on activity.

Decreases in the skin carcinoma incidence observed with 3-ethyl- and 3-t-butyl-TCQ as compared with 3-methyl-TCQ afford further support for the hypothesis that activity in TCQ and its derivatives is controlled by stereochemical factors related to the coplanar area of the molecule. Comparative reassessment of the activities of all known TCQ derivatives and analogues implies a highly specific orientation of the carcinogen at the tissue receptor.

PREVIOUS studies on the influence of substitution in the peripheral rings of the epidermal carcinogen tricycloquinazoline (TCQ, I) showed that of the four possible monomethyl derivatives, only one, 2-methyl-TCQ, was inactive.[1] Moreover, the 2-hydroxy and 2-methoxy derivatives were also virtually inactive.

This loss of carcinogenicity results, it has been suggested, from the interference of the 2-substituent with carcinogen-tissue receptor interaction. Possible interactions can involve either covalent bonding or multiple weak bonding to a structurally specific receptor. Covalent bonding appeared less likely, since TCQ was not found to be strongly bound to mouse skin protein at a level comparable to that observed with other epidermal carcinogens.[2]

The trigonal symmetry of TCQ permits two sets of three identical modes of union with a receptor. In mono-substituted derivatives, these three modes are not identical even though two-thirds of the molecular shape is unchanged. Since 2-methyl-TCQ is inactive, the substituent must affect all three modes and from this it was deduced that there is most probably a three-point union between TCQ and the receptor. The substituent would than influence the stereochemical fit of the whole molecule, rather than act as a blocking group at one fixed position. The greatest steric hindrance would

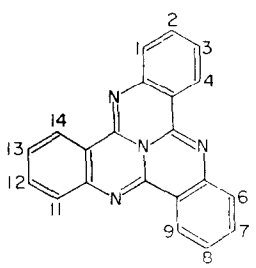
be provided by a 2-substituent, the magnitude of the hindrance being controlled by the size of the substituent.

Because of its small size, a fluoro substituent will not significantly alter the overall size and shape of the TCQ molecule, although it will block any direct interaction with a tissue receptor through the substituted position. Hence the carcinogenicity of 2-fluoro-TCQ has been examined to determine more directly the involvement of the 2-position in TCQ carcinogenesis. The carcinogenicity of 2-methyl-TCQ following repeated skin painting has been re-assessed, and it has been examined also for initiating action with subsequent croton oil treatment. Additionally, several 3-substituted derivatives have been examined for comparison with the influence of 2-substitution.

EXPERIMENTAL

Preparation of compounds

New tricycloquinazolines and intermediates are given in Table 1. The following adaptations of previously reported procedures[3] were employed in their synthesis.



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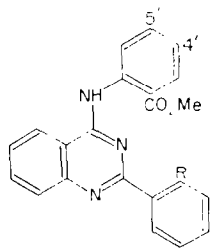

(II, R = NO_2; III, R = NH_2)

4-t-Butylhydroxyiminoacetanilide. To a stirred solution of chloral hydrate (33·7 g) and hydrated sodium sulphate (75 g) in water (2250 ml) was added 4-amino-t-butylbenzene[4] (28 g) dissolved in N-hydrochloric acid (200 ml) followed by hydroxylamine hydrochloride (41·2 g) in water (300 ml). The solution was slowly warmed to 50–60° and kept at that temperature for 24 hr; solidification of the oil which separated was followed by the separation of crystals (30 g). Recrystallisation from light petroleum furnished the pure *hydroxyimino derivative*, m.p. 149–150° (Found:C, 65·5; H, 7·0; N, 12·4. $C_{12}H_{16}N_2O_2$ requires C, 65·4; H, 7·3; N, 12·7%). A further quantity (2·6 g) was isolated after the mother liquor from the reaction mixture had been kept at 50–60° for 40 hr. When this reaction was conducted at a higher temperature, an intractable tar was obtained.

5-t-Butylisatin. 4-t-Butylhydroxyiminoacetanilide (30 g) was gradually added to sulphuric acid (75% v/v, 130 ml) to maintain the reaction temperature at 60–70°. After being heated at 80° a further 15 min, the mixture was poured on to ice (1500 g). A solution of the precipitate in aqueous sodium hydroxide (33%, 36 ml) was acidified with hydrochloric acid to obtain a small precipitate, filtered, and acidified to Congo red. The precipitated *isatin* (25·3 g, m.p. 150–152°) had m.p. 155–156° after crystallisation from acetic acid and then from light petroleum (Found: C, 70·7; H, 6·8; N, 6·8. $C_{12}H_{13}NO_2$ requires C, 70·9; H, 6·5; N, 6·9%). Buu-Hoi and Geuttier[5] describe this compound as an orange tar.

Anthranilic Acids A. The isatin derivative (0·075 mole), dissolved in aqeuous

TABLE 1.

Compound	Method and yield	m.p.	Found (%) C	H	N	Formula	Required (%) C	H	N
Anthranilic acids									
5-*Ethyl*	A, 75%	129–131°	65·2	7·2	8·6	$C_9H_{11}NO_2$	65·4	6·7	8·5
5-*t-Butyl*	A, 93	153–154	68·7	7·7	7·1	$C_{11}H_{15}NO_2$	68·4	7·8	7·3
Methyl anthranilates									
5-*Ethyl*	B, 44	87–89			7·9	$C_{10}H_{13}NO_2$			7·8
5-*t-Butyl*	B, 95	97–98	69·5	8·6	7·0	$C_{12}H_{17}NO_2$	69·5	8·3	6·8
5-*Methoxy*	B, 66	35–36*	59·9	6·2	8·0	$C_9H_{11}NO_3$	59·7	6·1	7·7
4-*Fluoro*	B, 64	67–68**	57·0	4·6	8·2	$C_8H_8FNO_2$	56·8	4·7	8·3
4-(*Substituted-2′-methoxycarbonylanilino*)-2-o-*nitrophenylquinazolines* (II)									
4′-*Ethyl*	C, 87	170–171	66·9	5·1	12·6	$C_{24}H_{20}N_4O_4$	67·3	4·7	13·1
4′-*t-Butyl*	C, 91	169–170	68·4	5·3	12·2	$C_{26}H_{24}N_4O_4$	68·4	5·3	12·3
4′-*Methoxy*	C, 86	181–182·5	64·5	4·3	12·8	$C_{23}H_{18}N_4O_5$	64·2	4·2	13·0
5′-*Fluoro*	C, 85	219–221	63·4	3·6	13·3	$C_{22}H_{15}FN_4O_4$	63·1	3·6	13·4
4-(*Substituted-2′-methoxycarbonylanilino*)-2-o-*aminophenylquinazolines* (III)									
4′-*Ethyl*	D, 81	166–167			13·9	$C_{24}H_{22}N_4O_2$			14·1
4′-*t-Butyl*	D, 90	206–207	73·3	6·1	13·0	$C_{26}H_{26}N_4O_2$	73·2	6·1	13·1
4′-*Methoxy*†	D, 86	181–182	69·8	5·2		$C_{23}H_{20}N_4O_3$	70·0	5·0	
5′-*Fluoro*‡	D, 82	229–230	68·1	4·3	14·3	$C_{22}H_{17}FN_4O_2$	68·1	4·4	14·4
Substituted tricycloquinazolines (I)									
3-*Ethyl*	E, 87	256–257	79·0	4·5	16·0	$C_{23}H_{16}N_4$	79·3	4·6	16·1
3-*t-Butyl*	E, 80	204–205	80·0	5·5	14·8	$C_{25}H_{20}N_4$	79·8	5·4	14·9
3-*Methoxy*	E, 62	239–240	75·3	4·1	15·7	$C_{22}H_{14}N_4O$	75·4	4·0	16·0
2-*Fluoro*	E, 68	330–332	74·4	3·2	16·6	$C_{21}H_{11}FN_4$	74·6	3·3	16·6

* B.p. 159–161°/17 mm
** B.p. 128–130°/16 mm
† Its *acetyl derivative* had m.p. 284–286° (Found: C, 67·8; H, 5·0. $C_{25}H_{22}N_4O_4$ requires C, 67·9; H, 5·0%).
‡ Its *acetyl derivative* had m.p. 254–255° (Found: N, 13·0. $C_{24}H_{19}FN_4O_3$ requires N, 13·0%).

sodium hydroxide (10%, 125 ml), was treated dropwise during 30 min with hydrogen peroxide (30%, 25 ml), then heated to 90° for 15 min, cooled, and acidified to pH 4·5–5. The anthranilic acid which separated was crystallised from light petroleum.

Methyl Anthranilates B. A mixture of the anthranilic acid (1·16 mole), methanol (36 ml) and concentrated sulphuric acid (18 ml) was heated at 100° under reflux for 2 hr and then poured with stirring on to a slurry of potassium carbonate (54 g) and crushed ice (300 g). The precipitated ester was dried and distilled or crystallised from light petroleum or aqeuous methanol.

4-(*Substituted-2′-methoxycarbonylanilino*)-2-*o-nitrophenylquinazolines* (II) *C.* 4-Chloro-2-*o*-nitrophenylquinazoline[3] (5·72 g), the substituted methyl anthranilate (0·022 mole), acetone (200 ml) and hydrochloric acid (0·2 ml) were heated together under reflux for 2 hr. The solid which separated overnight was suspended in hot methanol (200 ml) and basified with aqueous ammonia; water (100 ml) was added to give the required nitrophenylquinazoline, which was crystallised from ethanol or butanol.

4-(*Substituted-2′-methoxycarbonylanilino*)-2-*o-aminophenylquinazolines* (III) *D.* The above nitrophenylquinazoline (0·015 mole) was dissolved in boiling butanol (200–500 ml) and treated at 90–95° with hydrazine hydrate (10 ml) and small amounts of Raney nickel to maintain a steady evolution of nitrogen. When nitrogen ceased to be evolved, the mixture was boiled, filtered, and concentrated to yield the required aminophenylquinazoline, which was crystallised from ethanol or butanol.

Substituted Tricycloquinazolines (I) *E.* The foregoing aminophenylquinazoline (0·01 mole) was added to a solution of phosphorus pentoxide (26 g) in phosphoric acid (d, 1·75, 100 g) and the mixture was heated at 165–170° for 3 hr. Water (300 ml) and kieselguhr (Hyflo-Supercel, 15 g) were added; the mixed solids were collected, washed free from acid with water, dried, and continuously extracted with toluene (200 ml). Evaporation of the toluene extract yielded crude material from which the required tricycloquinazoline was isolated by boiling for 2 hr in turn with 6N-hydrochloric acid (200 ml) and 4N-sodium hydroxide (200 ml) and crystallisation from toluene (light petroleum for 3-t-butyltricycloquinazoline).

Each tricycloquinazoline derivative was further characterised by the close similarity of its absorption spectrum to that of tricycloquinazoline[3] and by its uniform behaviour on thin-layer chromatography on silica-gel.

Determination of carcinogenic activity

Young adult male albino mice of a random bred strain (Schofield) were employed in all tests. They were maintained in groups of 20–25 on a standard cubed diet with water *ad libitum*. Dorsal hair was removed with electric clippers at the beginning of each test and then subsequently when necessary.

For tests on direct skin carcinogenicity, compounds were applied in benzene solution (0·3 ml) at a concentration of 1 mg/ml twice weekly for 50 weeks. Mice were then examined weekly for tumours until the experiments were terminated and were killed when they were ill or when tumours were considered malignant. All tumours were taken for histological examination and tumour incidences were assessed from the number of mice surviving (at risk) when tumours were first observed.

In assessing initiating action, compounds were applied in benzene (1 mg/ml) to the clipped dorsal skin four times at twice weekly intervals (total dose 1·2 mg). Four

weeks after the last application, the mice received twice weekly skin painting of 5 per cent v/v croton oil in liquid paraffin for 20 weeks. The number of benign papillomas on the treated dorsal skin was recorded weekly until the experiments were terminated.

RESULTS

The previously reported inactivity of 2-methyl-TCQ following twice weekly skin painting has been confirmed since skin papillomas developed in only 2 (4 per cent) of treated mice (Table 2). Skin papillomas were observed in a similar number of control mice treated with benzene (Table 2). Moreover, 2-methyl-TCQ was shown to be without initiating action since the incidence of skin papillomas following croton oil treatment for 20 weeks did not differ significantly from that in control, benzene treated mice (Table 3).

In contrast, 2-fluoro-TCQ proved to be carcinogenic following repeated skin painting, its activity assessed from the final skin tumour incidence being comparable to that of TCQ and 3-fluoro-TCQ[1] (Table 2). Moreover, the rate of skin tumour

TABLE 2. SKIN TUMOUR INCIDENCES IN MICE TREATED WITH TRICYCLOQUINAZOLINE AND RING SUBSTITUTED DERIVATIVES

Compound	Number of mice at risk	Duration of experiment (days)	Skin tumour incidence			
			Total tumours		Skin carcinomas	
			Number	Percentage	Number	Percentage
TCQ	36	471	29	81	27	75
2-Methyl-TCQ	48	490	2	4	0	—
2-Fluoro-TCQ	56	454	44	79	13	23
3-Methoxy-TCQ	50	464	6	12	0	—
3-Ethyl-TCQ	58	538	32	55	14	24
3-t-Butyl-TCQ	56	544	30	54	11	20
3,8-Difluoro-TCQ	35	555	14	40	4	11
Benzene (solvent controls)	28	654	1	4	0	0

TABLE 3. INDUCTION OF PAPILLOMAS IN MOUSE SKIN BY TCQ OR SUBSTITUTED DERIVATIVES AND CROTON OIL.

Compound	Number of mice	Survivors at end of croton oil treatment (20 weeks)	Skin papilloma incidence percentage	Average number of papillomas per survivor
TCQ	25	23	66	2·2
2-Methyl-TCQ	25	22	9	1·5
2-Fluoro-TCQ	25	24	44	2·4
3-Fluoro-TCQ	25	24	36	1·8
Benzene	25	25	8	1·0

development in 2-fluoro-TCQ treated mice did not differ greatly from that observed with TCQ (Fig. 1). However, a large proportion of the skin tumours induced with 2-fluoro-TCQ were benign papillomas and the incidence of skin carcinomas (23%) is significantly less than that induced with TCQ (75 %) or 3-fluoro-TCQ (61%).

2-Fluoro-TCQ had no unusual toxic properties and mice tolerated skin painting with this compound as well as with TCQ. Thus the survival rates after 40 weeks treatment with TCQ and 2-fluoro-TCQ were 62% and 57% respectively. Hence the low skin carcinoma incidence induced with 2-fluoro-TCQ compared with TCQ is not simply due to low survival from an increased toxicity of the compound but represents a difference in carcinogenic activity.

2-Fluoro-TCQ was also active as an initiator, skin papillomas developing in 48% of mice following four applications of the compound (total dose 1·2 mg.) and subsequent croton oil treatment for 20 weeks. This activity is lower than that observed with TCQ (Table 3).

The finding that 3-methoxy-TCQ is only weakly active inducing skin papillomas in 12% of mice at risk (Table 2) implies that the low activity of 2-methoxy-TCQ[1] cannot be cited as evidence implicating the 2-position in carcinogenesis. Evidently a methoxyl substituent in TCQ greatly reduces carcinogenicity irrespective of its position. Whether this inhibition represents a specific effect due to structural modification or simply to a greater susceptibility of this substituent to metabolism is still unresolved.

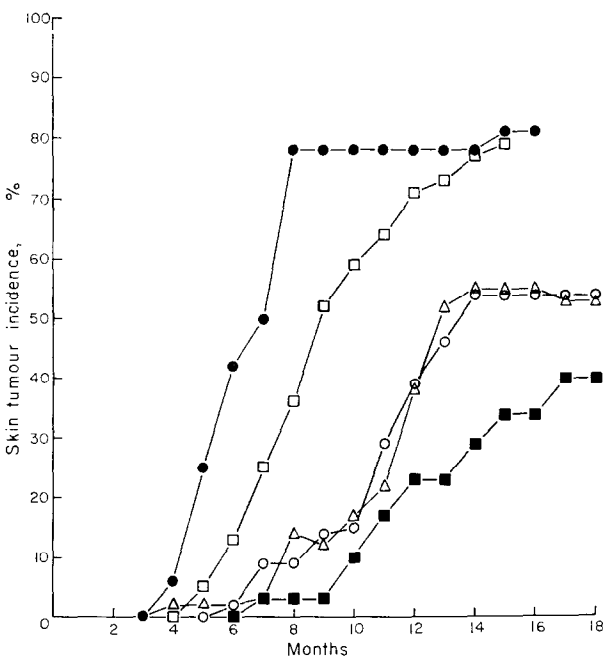

FIG. 1. Development of skin tumours in mice treated with TCQ or ring substituted derivatives. TCQ ●, 2-Fluoro-TCQ □, 3-Ethyl-TCQ △, 3-t-Butyl-TCQ ○, 3,8-Difluoro-TCQ ■.

Although 8-methoxy-3,4-benzopyrene is one of the most potent carcinogens known,[6] the influence of methoxyl substitution in polycyclic hydrocarbon carcinogens is variable. Thus enhancement and depression of carcinogenicity have been observed following methoxyl substitution and these effects are probably related to the ease with which the compounds undergo metabolism.

Fluoro substitution at the 3-position in TCQ previously was shown to reduce

carcinogenicity as assessed from repeated skin painting.[1] The influence of a 3-fluoro substituent is further emphasized by the finding that 3-fluoro-TCQ was less active than TCQ as an initiator. (Table 3). Moreover introduction of an additional fluoro substituent at a position equivalent to the 3-position brings about a further reduction in carcinogenic activity. Hence repeated skin painting with 3,8-difluoro-TCQ induced skin tumours in 40% of mice at risk (Table 2) compared with skin tumour incidences[1] of 81% and 76% following treatment with TCQ and 3-fluoro-TCQ respectively. The decrease in carcinogenicity is also revealed by a longer latent period for tumour induction (Fig. 1) and more particularly by the lower skin carcinoma incidence. Hence with 3,8-difluoro-TCQ, skin carcinoma developed in only 4 mice (11%) compared with incidences of 61 and 75 % following treatment with 3-fluoro-TCQ and TCQ respectively. Although less pronounced, this effect is comparable to that previously observed following methyl substitution at the 3- and equivalent 8 and 13-positions.[1]

Previous studies indicated that substitution at the 3-position results in a progressive loss of carcinogenicity with increasing size of substituent up to methyl.[1] When assessed from the total skin tumour incidences, larger 3-substituents have not resulted in further losses in activity. Hence 3-ethyl-TCQ and 3-t-butyl-TCQ have activities comparable to that of 3-methyl-TCQ (Table 2). Moreover, the rates of tumour development in mice treated with these derivatives were closely similar (Fig. 1). However, when assessed from the skin carcinoma incidences, the 3-ethyl substituent was found to depress activity (Table 2). Thus the skin carcinoma incidence in mice treated with 3-ethyl-TCQ (24%) was significantly lower than that induced with 3-methyl-TCQ (48%). Whilst 3-t-butyl-TCQ was also less active than 3-methyl-TCQ when assessed from skin carcinoma incidences, its activity (20%) is not significantly different from that of 3-ethyl-TCQ.

DISCUSSION

The finding that 2-fluoro-TCQ has appreciable carcinogenic activity provides convincing evidence that direct covalent bonding of carcinogen to a tissue receptor through the 2-position is not involved in carcinogenesis. Hence the loss of activity following 2-methyl substitution cannot be ascribed to blocking of direct interactions through this position, but more likely can be interpreted in terms of interference of stereochemical fit of carcinogen at a cell receptor. This hypothesis also explains more satisfactorily why 2-methyl substitution abolishes activity when, on account of the trigonal symmetry of TCQ, there are two other equivalent ring positions (viz. 7 and 12). The influence of substituents at other ring positions further supports such a concept whilst the virtual inactivity of iso-TCQ and other structural analogues[7] emphasizes the importance of molecular shape and size for carcinogenicity.

Previously, it was demonstrated that substitution at the 3-position resulted in a progressive loss of carcinogenic activity as the van der Waals radius of the substituent increased from H through F and Br to methyl (1·1Å to 2·0Å).[1] This correlation is further emphasized by the finding that the carcinogenic activity of 3-ethyl-TCQ is significantly lower than that of 3-methyl-TCQ. Moreover there is a further but less marked loss of activity in the 3-t-butyl-TCQ derivative. In view of the large molecular volume of the 3-t-butyl group, this substituent may have been expected to diminish carcinogenic activity more than was observed. However despite its greater van der

Waals volume, a t-butyl group produces no greater extension of the co-planar area of the substituted TCQ than does an ethyl group. The influence of the proximity of these substituents on the involvement of the 2-position in carcinogenesis cannot be simply assessed but the biological evidence indicates that it is not great.

Whilst methyl substitution at the 3-position in TCQ only partially depresses carcinogenicity, introduction of an additional methyl group at the equivalent 8-position brings about a further more marked loss of activity.[1] The present findings demonstrate a similar effect with fluoro substituents at the 3- and equivalent positions, the carcinogenic activities of TCQ, 3-fluoro-TCQ and 3,8-difluoro-TCQ being 75, 61 and 11% respectively. These results emphasize that the carcinogenic activity of TCQ is much more sensitive to the number of 3-substituents rather than the overall molecular size of a single substituent. For example, 3,8-difluoro-TCQ (carcinoma incidence, 11%) is much less active than 3-ethyl-TCQ (28%). This, it is suggested, may be interpreted in terms of a highly specific orientation of the carcinogen at a cell receptor which involves at least two of the peripheral benzene rings. Moreover, the marked differences in the influence of substituents at the 2- and 3-positions in TCQ imply that the specific orientation of TCQ at the cell receptor is critically controlled by the 2- and equivalent positions although they are not directly involved in tissue bonding.

Modification of carcinogenicity ascribable to stereochemical factors has been reported in a number of studies with other polycyclic hydrocarbons. Thus in 1,2-benzanthracene, there is a general decrease in carcinogenicity when methyl groups at favourable positions are replaced by larger groups. as evidenced by the inactivity of 10-ethyl-1,2-benzanthracene.[8] Similarly lengthening the alkyl substituent beyond methyl at the 10-position in 3-fluoro-5,6-benzacridine abolishes sarcomogenic activity.[9] These results however reflect the decrease in effectiveness by chain lengthening in an alkyl substituent which introduces activity into an inactive parent hydrocarbon and as such are not directly comparable with the present findings. More relevant data have been reported by Miller and Miller who showed that fluoro substitution at the 3′ and possibly 4′ positions in 10-methyl-1,2-benzanthracene reduced skin carcinogenicity.[10] The influence of other substituents at these positions in 10-methyl-1,2-benzanthracene has not been reported but these findings are in agreement with the observation that introduction of a second methyl substituent into the angular ring of highly carcinogenic monomethyl-1,2-benzanthracenes causes partial or total loss of activity (cf. 10-methyl) and 1′,10-dimethyl-1,2-benzanthracene).[8]

Recent studies utilizing C^{14}-TCQ of high specific activity have demonstrated a very low level of bound radioactivity in mouse skin equivalent to 2×10^{-4} μmole TCQ/100 mg dried skin. Hence, the possibility that covalent bonding of carcinogen to epidermal protein may be involved in TCQ carcinogenesis cannot be excluded. However, the level of TCQ binding is very much lower than that observed with other epidermal carcinogens and perhaps more significantly, it is much less than that detected with inactive hydrocarbons such as phenanthrene.

The sensitivity of TCQ carcinogenicity to changes in chemical structure, the requirement of planarity and the influence of peripheral ring substituents, it is considered, may be interpreted more satisfactorily in terms of multiple low energy bonding with a tissue receptor. Moreover, the sensitivity of activity to structural change in the carcinogen implies also a highly specific structural specificity in the tissue receptor. Since TCQ and the active 3-methyl-TCQ derivative have been shown[11] to interfere

with DNA breakdown in skin whereas the inactive 2-methyl-TCQ was without effect, this suggests that interactions with DNA may be involved. Such interactions could occur by weak bonding of TCQ to the planar bonded purine and pyrimidine pairs in DNA. Bonding by overlap of π-orbitals is provided for by the six aromatic rings in TCQ and the aromatic rings of the purines and pyrimidines. Augmentation of this binding could then result from hydrogen bonding to the three peripheral nitrogen atoms in TCQ.

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