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Intramolecular Synergism, an Explanation for the Enhanced Fungitoxicity of Halo-8-quinolinols

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Summary. An antifungal study against *Aspergillus niger, A. oryzae, Myrothecium verrucaria,* and *Trichoderma viride* in Yeast Nitrogen Base supplemented with 1% *D*-glucose and 0.088% *L*-asparagine was carried out using 8-quinolinol and 3-, 5-, 6-, 7-, 3,6-, and 5,7-chlorinated and brominated-8-quinolinols. Binary mixtures of 3- and 6-halo- and 5- and 7-halo-8-quinolinols were intermolecularly synergistic. MICs of the monohalo synergistic mixtures admixed with a MIC of the corresponding dihalo-8-quinolinols were not synergistic. The dihalo-8-quinolinols with substituents in positions corresponding to those of the synergistic binary mixtures appeared to attack the same sites of action as the binary pairs. The enhanced activities of 3,6- and 5,7-dichloro-8-quinolinols and 3,6- and 5,7-dibromo-8-quinolinols are believed to be due to intramolecular synergism. The greater fungitoxicity of 5-, 6-, and 7-monohalo-8-quinolinols over 8-quinolinol can also be explained as due to intramolecular synergism. 3,6-Dihalo- and 5,7-dihalo-8-quinolinols formed synergistic pairs of compounds.

Keywords. Antifungal activity: Synergism; Intramolecular synergism; Intramolecular synergism; 3,6-Dichloro- and 5,7-dichloro-8-quinolinols; 3,6-Dibromo- and 5,7-dibromo-8-quinolinols.

Intramolekularer Synergismus als Erklärung für die erhöhte Fungitoxizität von halogenierten 8-Chinolinolen

Zusammenfassung. 8-Chinolinol und verschiedene halogenierte 8-Chinolinole wurden auf ihre Fungitoxizität gegenüber *Aspergillus niger, A. oryzae, Myrothecium verrucaria* und *Trichoderma viride* untersucht. Binäre Mischungen von 3- und 6-Halogen- bzw. 5- und 7-Halogen-8-chinolinol zeigten intermolekularen Synergismus, während bei Mischungen von mono- und dihalogenierten 8-Chinolinolen kein entsprechender Effekt beobachtet werden konnte. Die erhöhte Aktivität von 3,6- und 5,7-Dichlor-8-chinolinol und 3,6- und 5,7-Dibrom-8-chinolinol wird durch intramolekularen Synergismus erklärt, desgleichen die höhere Aktivtät monohalogenierter 8-Chinolinole gegenüber 8-Chinolinol. 3,6-Dihalogenierte und 5,7-dihalogenierte 8-Chinolinole bilden synergistische Paare.

Introduction

In our attempts to understand the mode of fungitoxicity of 8-quinolinol and its halogenated derivatives, it was observed that the mechanisms of action of 8-quinolinol and its 5-iodo analogue were different. Thiol-containing compounds (*L*-cysteine, cysteamine, glutathione, and N-acetyl-*L*-cysteine) reversed the inhibi-

tory action of 8-quinolinol and not that of 5-iodo-8-quinolinol [1]. It was further observed that the two compounds formed a synergistic mixture [2]. The study of synergism was extended to include 8-quinolinol and its 5- and 7-fluoro, chloro, bromo, and iodo analogues. The possibility of reversing fungitoxicity with *L*-cysteine was also included. Binary mixtures of 8-quinolinol, 5-fluoro-, and 7-fluoro-8-quinolinols showed additive activity, and their respective toxicities were reversed by *L*-cysteine. These results suggested a common mode of action for the three toxicants. 8-Quinolinol in binary combination with its 5- and 7-chloro, bromo, and iodo analogues resulted in potentiated fungitoxic action. Potentiation of fungitoxicity was also observed with binary mixtures of any combination of 5-chloro-, bromo-, and iodo-8-quinolinols in combination with the 7-chloro, bromo, and iodo analogues. *L*-Cysteine did not protect the fungi from these toxicants. This suggested that the sites of action of these compounds were different from each other and from 8-quinolinol and its 5- and 7-fluoro analogues [3]. The study was further extended to include 2-, 3-, 4-, 5-, 6-, and 7-chloro- and bromo-8-quinolinols. 8-Quinolinol and its 2-, 3-, and 4-chloro and bromo analogues formed additive mixtures, and their toxicities were reversed by *L*-cysteine or N-acetyl-*L*-cysteine. The toxicities of 5-, 6-, and 7-chloro- and bromo-8-quinolinols were not reversed by *L*-cysteine and formed synergistic mixtures with each other and with 2-, 3-, and 4-chloro- and bromo-8-quinolinols. 8-Quinolinols with chloro or bromo substituents on the phenol ring acted at different sites from each other and from the quinolinols with the same substituents on the pyridine ring. The 2-substituted 8-quinolinols possessed poor to no activity under the test conditions. Mixtures of 5-chloro- and 5-bromo-, 6-chloro- and 6-bromo, and 7-chloro- and 7-bromo-8-quinolinols showed additive fungitoxicity, indicating that the geometries of the binding sites of action are not so constrained that they cannot accommodate analogously substituted chloro- and bromo-8-quinolinols [4].

Since single substituents on the phenol ring of 8-quinolinol changed the sites of action of the compound, multiple substituents on the phenol ring of 8-quinolinol should allow the compound to attack the various sites simultaneously, for which each substituent showed a specificity. When two singly substituted 8-quinolinols form a synergistic mixture, this is called intermolecular synergism. When a single compound contains the substituents in the analogous positions to those of the singly substituted compounds and possesses enhanced activity over that of the singly substituted compounds, it can be considered as due to intramolecular synergism.

A review of our earlier work revealed that, in general, the 5,7-dichloro- and dibromo-8-quinolinols were more fungitoxic than the corresponding 5- and 7-halo analogues [5]. This was especially striking among the chloro and bromo analogues of the 5-, 7-, and 5,7-substituted 2-methyl-8-quinolinols. 2-Methyl-8-quinolinol possessed poor antifungal activity [6].

The expression "intramolecular synergism" came to our attention earlier [7]. The author claimed to have prepared mixed ligand chelates containing two or more potentially bioactive compounds bound by a metal. The authenticity of the proposed structures was not fully established, and no data on dissociation of the chelates were reported. It was not even demonstrated that the materials were single compounds and not mixtures. If antifungal activity was observed, it was attributed to intramolecular synergism. The term intramolecular synergism was reported again

more recently [8]. Cellulase A from *Cellomonas firmi* is composed of a catalytic domain and a nonhydrolytic cellulose-binding domain that function independently. The individual domains interact in the disruption of and hydrolysis of cellulose from cotton fibers. This was called intramolecular synergism [9].

The present work is concerned with the development of the concept of intramolecular synergism on a more rigorous basis. The approach in this study is based on the presence or absence of synergism between a pair of singly substituted compounds and the correspondingly disubstituted compound. As an example, 5- and 7-chloro-8-quinolinols form a synergistic pair of fungitoxicants [3]. If the minimal inhibitory concentration (MIC) of the binary mixture is admixed with a MIC of 5,7-dichloro-8-quinolinol and further synergism is observed, it will be considered that the sites of action of the components of the ternary mixture are different, and the synergism is intermolecular. If the ternary mixture exhibits no further synergism, it will be considered that the binary mixture acts at the same sites as the compound disubstituted in the corresponding positions as the components of the binary mixture. The activity of the disubstituted compound is then considered as due to intramolecular synergism.

The approach to the determination of intramolecular synergism among this class of compounds was carried out as indicated above. MICs of binary mixtures of 3- and 6-chloro- and 3- and 6-bromo-8-quinolinols were tested for antifungal activity admixed with MICs of the corresponding 3,6-dichloro- and dibromo-8- quinolinols. Similar testing of 5- and 7-chloro and 5- and 7-bromo-8-quinolinols together with the corresponding dichloro- and dibromo-8-quinolinols was carried out. MICs of binary mixtures of 5-, 6-, and 7-chloro- and bromo-8-quinolinols with 8-quinolinol mixed with MICs of the corresponding 3,6- and 5,7-dihalo-8-quinolinols were also tested. The fungi included *A. niger, A. oryzae, M. verrucaria*, and *T. viride*, and the growth medium employed was Yeast Nitrogen Base supplemented with *D*-glucose and *L*-asparagine.

Results and Discussion

MICs of 8-quinolinol, the 3-, 5-, 6-, and 7-chloro- and bromo-8-quinolinols, and 3,6-and 5,7-dichloro- and dibromo-8-quinolinols against the four fungi are summarized in Table 1. Data resulting from the search for synergism between the binary mixtures of 8-quinolinol and 5-, 6-, and 7-chloro- and bromo-8-quinolinols, 3-chloro- and bromo-8-quinolinol and the respective 6-chloro- and bromo-8-quinolinols, 5-chloro- and bromo-8-quinolinols and the respective 7-chloro- and bromo-8-quinolinols are given in Table 2. Also included are the results obtained from the mixtures of 3,6-dichloro- and 5,7-dichloro-8-quinolinols and 3,6-dibromo- and 5,7-dibromo-8-quinolinols. Ternary mixtures composed of MICs of the synergistic mixture of 3- and 6-chloro-8-quinolinols and the MIC of 3,6-dichloro-8-quinolinol, the mixture of the corresponding bromo-8-quinolinols and similar mixtures of 5- and 7-chloro- and 5,7-dichloro-8-quinolinols and the corresponding bromo analogues are also listed. The last set of mixtures in Table 2 includes MICs of mixtures of 8-quinolinol and 5-, 6-, and 7-chloro- and bromo-8-quinolinols admixed with the MICs of the corresponding 3,6- or 5,7-dichloro- or dibromo-8-quinolinols.

Table 1. Minimal antifungal activity of 3-, 5-, 6-, 7-, 3,6-, and 5,7-chloro and bromo-8-quinolinols in Modified Yeast Nitrogen Base[a] at 28 °C in shake culture after six days (mmol/l(μg/ml))

No.	Compound[b]	*A. niger*	*A. oryzae*	*M. verrucaria*	*T. viride*
1.	8-quinolinol	0.055 (8)	<0.0069 (<1)[c]	0.014 (2)	0.17 (24)
2.	3-Chloro-8-quinolinol	0.033 (6)	0.033 (6)	0.017 (3)	0.066 (12)
3.	3-Bromo-8-quinolinol	0.031 (7)	0.031 (7)	0.013 (3)	0.052 (12)
4.	5-Chloro-8-quinolinol	0.011 (2)	0.011 (2)	<0.0056 (<1)	0.033 (6)
5.	5-Bromo-8-quinolinol	0.013 (3)	0.013 (3)	0.013 (3)	0.026 (6)
6.	6-Chloro-8-quinolinol	0.017 (3)	0.011 (2)	<0.0056 (<1)	0.022 (4)
7.	6-Bromo-8-quinolinol	0.0089 (2)	0.0089 (2)	<0.0045 (<1)	0.018 (4)
8.	7-Chloro-8-quinolinol	0.011 (2)	0.011 (2)	<0.0056 (<1)	0.011 (2)
9.	7-Bromo-8-quinolinol	0.0089 (2)	0.013 (3)	0.0089 (2)	0.013 (3)
10.	3,6-Dichloro-8-quinolinol	<0.0047 (<1)	<0.0047 (<1)	<0.0047 (<1)	0.014 (3)
11.	3,6-Dibromo-8-quinolinol	0.023 (7)	0.0099 (3)	0.0066 (2)	0.26 (78)
12.	5,7-Dichloro-8-quinolinol	0.0093 (2)	<0.0047 (<1)	<0.0047 (<1)	0.0093 (2)
13.	5,7-Dibromo-8-quinolinol	0.0066 (2)	0.0066 (2)	<0.0033 (<1)	0.0099 (3)

[a] Medium enriched with 1% *D*-glucose and 0.088% *L*-asparagine; [b] the data for compounds 1–9 were taken from Ref. [4]; [c] the symbol < indicates inhibitory at under 1 μg/ml (lowest level tested)

M. verrucaria was not employed in the studies shown in Table 2 because at least one component of each mixture was inhibitory to the fungus at < 1 μg/ml, the lowest level of the compound tested. Complete inhibition at < 1 μg/ml is not considered a MIC. Twenty-two mixtures were considered for testing for synergism against *A. niger*, *A. oryzae*, and *T. viride*. Thus, of the 66 cases, 19 could not be tested because at least one component of the mixture inhibited the fungus at < 1 μg/ml. All binary combinations tested as shown in Table 2 were synergistic, as indicated by complete inhibition of growth of the inoculum at 40% or less of the mixture. Of the ternary mixtures that could be tested, all caused complete inhibition at 50% of the mixture and were not synergistic. It is of special interest to note that the binary mixtures composed of 3,6-dichloro- and 3,6-dibromo-8- quinolinols formed synergistic combinations with the corresponding 5,7-dichloro- and 5,7-dibromo-8-quinolinols.

It has long been known that 5,7-dichloro-8-quinolinol [5] and its 2-methyl analogue [6] were especially active antifungal agents. Systematic studies of the fungitoxicity of the monohalogenated 8-quinolinols showed that halogens on the phenol ring, larger than fluorine, affected different sites of action. When the pyridine ring was similarly halogenated, the mechanism(s) of action did not appear to be different from that of 8-quinolinol. That fact that *L*-cysteine protected the fungi from the toxicity of 8-quinolinol, its 5- and 7-fluoro analogues and the analogues halogenated in the pyridine ring and not against the 8-quinolinols with the larger halogen substituents on the phenol ring, was consistent with the observation that altering the geometry of the phenol ring of 8-quinolinol affected different sites of action [4]. Furthermore, synergistic binary mixtures were formed between 5-, 6-, and 7-halo-8-quinolinols and with 8-quinolinol.

It was reasonable to expect that if 3- and 6- and 5- and 7-halo-8-quinolinols formed synergistic mixtures, 3,6- and 5,7-dihalo-8-quinolinols should be able to

Table 2. Search for synergism between binary mixtures of 8-quinolinol and 5-, 6-, 7-chloro and bromo-8-quinolinols and between the corresponding 3- and 6-chloro- and bromo-, and 5- and 7-chloro- and bromo-8-quinolinols, and between ternary mixtures composed of the MICs of the binary mixtures and the MICs of the corresponding 3,6-dichloro- and dibromo- and 5,7-dichloro- and dibromo-8-quinolinols in Modified Yeast Nitrogen Base[a] at 28 °C in shake culture after six days

Mixtures of 8-Quinolinols	*A. niger*	*A. oryzae*	*T. viride*[b]
	Binary Mixtures		
5-Chloro + Unsubstituted	20[c]	NT[d]	40
5-Bromo + Unsubstituted	40	NT	40
6-Chloro + Unsubstituted	40	NT	40
6-Bromo + Unsubstituted	40	NT	40
7-Chloro + Unsubstituted	30	NT	40
7-Bromo + Unsubstituted	40	NT	40
3-Chloro + 6-Chloro	40	NT	40
3-Bromo + 6-Bromo	40	40	40
5-Chloro + 7-Chloro	30	NT	40
5-Bromo + 7-Bromo	30	30	40
3,6-Dichloro + 5,7-Dichloro	NT	NT	40
3,6-Dibromo + 5,7-Dibromo	20	30	30
	Ternary Mixtures[e]		
3-Chloro + 6-Chloro + 3,6-Dichloro	NT	NT	50
3-Bromo + 6-Bromo + 3,6-Dibromo	50	50	50
5-Chloro + 7-Chloro + 5,7-Dichloro	50	NT	50
5-Bromo + 7-Bromo + 5,7-Dibromo	50	50	50
5-Chloro + Unsubstituted + 5,7-Dichloro	50	NT	50
5-Bromo + Unsubstituted + 5,7-Dibromo	50	NT	50
6-Chloro + Unsubstituted + 3,6-Dichloro	NT	NT	50
6-Bromo + Unsubstituted + 3,6-Dibromo	50	NT	50
7-Chloro + Unsubstituted + 5,7-Dichloro	50	NT	50
7-Bromo + Unsubstituted + 4,7-Dibromo	50	NT	50

[a] Medium enriched with 1% *D*-glucose and 0.088% *L*-asparagine; [b] *M. verrucaria* was not employed as a test organism in this study because at least one component of each mixture was inhibitory to the fungus at < 1 µg/ml, the lowest level tested; this is not considered as a MIC; [c] percent of mixtures containing MICs of each toxicant causing 100% inhibition; [d] NT = not tested because at least one component of the mixture caused 100% inhibition of the fungus at < 1 µg/ml; this was the lowest level tested; [e] composed of the MIC of the binary mixture together with the MIC of the corresponding disubstituted 8-quinolinol

attack the same sites as the corresponding synergistic binary mixtures. If this were true, the disubstituted 8-quinolinols would represent examples of intramolecular synergism. The veracity of this concept was established by demonstrating the absence of further synergism between mixtures of the synergistic pairs of singly substituted 8-quinolinols and the corresponding disubstituted 8-quinolinols (Table 2). It is concluded that 3,6-dichloro-, 3,6-dibromo-, 5,7-dichloro-, and 5,7-dibromo-8-quinolinols owe their enhanced fungitoxicity to intramolecular synergism. In view of these concepts, the formation of synergistic mixtures composed of 3,6-dichloro- and 5,7-dichloro-8-quinolinols and of the corresponding bromo analogoues was to be expected (Table 2). A further consequence of this study is that the greater activity of the 5-, 6-, and 7-halo-8-quinolinols as compared with 8-quinolinol is also due to intramolecular synergism, as evidenced by the data on synergism between the phenol ring halogenated 8-quinolinols and 8-quinolinol (Tables 1, 2 [4]).

Experimental

8-Quinolinol, its 5-chloro, 5-bromo, 5,7-dichloro, and 5,7-dibromo analogues, *D*-glucose, and *L*-asparagine were purchased from Aldrich Chemical Company (Milwaukee, WI). The remaining 8-quinolinols were prepared according to methods found in the literature: 3-chloro, 3-bromo, 6-chloro, and 6-bromo [11], 7-chloro and 7-bromo [12], 3,6-dichloro and 3,6-dibromo [13]. Yeast Nitrogen Base was purchased from Difco Labs (Detroit, MI).

The test fungi included *Aspergillus niger* (ATCC 1004), *A. oryzae* (ATCC 1101), *Myrothecium verrucaria* (ATCC 9095), and *Trichoderma viride* (ATCC 8678). Synergism studies were conducted in Yeast Nitrogen Base supplemented with 1% *D*-glucose and 0.088% *L*-asparagine according to published methods [2–4]. MICs of the toxicants were obtained in μg/ml by serial dilution of the dimethyl sulfoxide (Me_2SO) solutions and recalculated to a molar basis for comparison. Synergism was sought by co-dissolving MIC levels of the toxicants in Me_2SO and incorporating them into the growth medium in 10% increments from 10 to 100% of the mixtures. Ternary mixtures composed of the MICs of the respective dehalo-8-quinolinols were tested for synergism in the same manner.

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[9] The usual definition of synergism is: the joint action of agents that when taken together increase each other's effectiveness [10]. This implies that both agents are effective and their effectiveness is greater than would be expected from additive action. In the case of the cellulase, hydrolytic activity is attributed only to one domain of the enzyme. It would require stretching the definition

of synergism to include this as synergism. Even though the binding domain enhances the activity of the hydrolytic domain, it has no hydrolytic capability itself.

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