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# Indole and (*E*)-2-Hexenal, Phytochemical Potentiators of Polymyxins against *Pseudomonas aeruginosa* and *Escherichia coli*

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**Combinations of polymyxins and phytochemicals were tested for antimicrobial activity against two gram-negative bacteria. Various degrees of potentiation were found against *Pseudomonas aeruginosa* and *Escherichia coli* with (*E*)-2-hexenal and indole. Three-compound combinations were found to further increase the activity of polymyxin B sulfate and colistin methanesulfonate against both bacteria. Combinations with colistin against *P. aeruginosa* resulted in the highest degree of potentiation, with a 512-fold increase in colistin antimicrobial activity. These results indicate the potential efficacy of phytochemical combinations with antibiotics to enhance total biological activity.**

In our continuing search for antimicrobial agents from plants, a number of secondary metabolites have been characterized as active principles. However, few phytochemicals have shown activity against gram-negative bacteria, especially the *Pseudomonas* species. (*E*)-2-Hexenal identified in cashew apple flavor (16) and indole in green tea flavor (12) are the rare phytochemicals that exhibit activity against *Pseudomonas aeruginosa* and *Escherichia coli.*

*P. aeruginosa* and *E. coli* are opportunistic pathogens that cause a variety of infections, usually in immunocompromised hosts. Polymyxins have been used for topical application with cultured skin on burns (3), for operative wound irrigation (5), for selective decontamination of the digestive tract (21), for superficial eye infections (20), and for the prevention of minor wound infections (2). Polymyxins are most often combined with other agents to provide broad-spectrum antibiotics, as with the classical neomycin sulfate and bacitracin combination, or potent narrow-spectrum antibiotics, as with tobramycin and fluoroquinolone for selective decontamination of the digestive tract. The low degree of absorption of polymyxins was originally an undesired trait as they could not be used internally. However, this trait is now advantageous because tissue impermeability reduces the toxicity to immunocompromised patients.

Polymyxins are polycationic peptide antibiotics which uniquely differ from other antibiotics by selectively targeting gram-negative bacteria. Polymyxin B is generally used in the sulfate form, while colistin, otherwise known as polymyxin E, is generally used in the methanesulfonate form (15). Colistin is less toxic but correspondingly less active; thus, polymyxin B tends to be more commonly used. Since they are most often used in combination with other antibiotics, it is important to assess the clinical potency of these combinations. It is also important to search for new and potentially superior combinations.

## MATERIALS AND METHODS

**Cultures.** The bacteria, *P. aeruginosa* ATCC 10145 and *E. coli* ATCC 9637, used for the experiment were purchased from the American Type Culture Collection (Rockville, Md.). NYG broth (0.8% nutrient broth [BBL], 0.5% yeast extract [Difco], 0.1% glucose) was used for the assay. *P. aeruginosa* and *E. coli* were inoculated into NYG broth and incubated without shaking for 48 h at 37°C prior to the antimicrobial activity assay.

**Chemicals.** Indole and (*E*)-2-hexenal were purchased from Aldrich Chemical Co. (Milwaukee, Wis.), and polymyxin B sulfate and colistin methanesulfonate were obtained from Sigma Chemical Co. (St. Louis, Mo.). For the experiment, all compounds except polymyxin B and colistin were first dissolved in *N*,*N*-dimethylformamide (DMF), which was purchased from EM Science (Gibbstown, N.J.).

**Determination of MICs and MBCs.** Broth macrodilution MICs were determined as previously described (16). Briefly, serial twofold dilutions of the test compounds were made in DMF, except the polymyxins, which were diluted in water, and 30 μl of each dilution was added to 3 ml of NYG broth. These were inoculated with 30 μl of a 2-day-old culture of either *P. aeruginosa* or *E. coli*. The cultures were incubated without shaking at 37°C for 48 h. The MIC was the lowest concentration of the test compound that demonstrated no visible growth.

The MBC was the lowest concentration of antibacterial compound that decreased the initial inoculum concentration by 99.9%. After the MIC was determined, 10-fold dilutions from each tube showing no turbidity were plated onto chemical-free NYG agar medium. After 1 day of incubation, MBC breakpoints were determined by using rejection values (19).

Combination studies were performed by a broth checkerboard method (18). A series of twofold dilutions of either polymyxin B or colistin were tested in combination with twofold dilutions of either indole or (*E*)-2-hexenal. In the three-compound combination studies, a single concentration of a third compound was added to the double-combination matrix. Assays were performed in triplicate on separate occasions. Subinhibitory amounts of (*E*)-2-hexenal and indole were included as confirmed by independent combination assays.

**Determination of FIC index.** The fractional inhibitory concentration (FIC) indices were calculated for both double (1, 7) and triple (1, 2) combinations from checkboard dilution data. The FICs for two-drug combinations were calculated as ($MIC_a$ combination/$MIC_a$ alone) + ($MIC_b$ combination/$MIC_b$ alone), where a and b were the two compounds used. For three-drug combinations, the FICs were calculated in the same way but with the addition of a third MIC fraction. For double combinations FIC indices of $\leq 0.5$, $>0.5$ to $<4$, and $\geq 4$ are considered synergistic, additive, and antagonistic, respectively. For triple combinations FIC indices of $\leq 1$, $>1$ to $<4$, and $\geq 4$ are considered synergistic, additive, and antagonistic, respectively.

**Growth curves.** Time-kill studies were performed to examine the effects of combinations of compounds in more detail. The culture tubes were prepared as described above and incubated at 37°C for 2 days. A 30-μl aliquot of the 48-h culture was inoculated into 3 ml of NYG broth containing appropriate concentrations of the test compounds. The initial population sizes were between $5 \times 10^6$ and $1 \times 10^7$ CFU/ml for *E. coli* and between $1 \times 10^6$ and $5 \times 10^6$ CFU/ml for *P. aeruginosa*. Samples were taken at selected times, and serial dilutions were made in sterile saline before the samples were plated onto NYG agar plates. The plates were incubated at 37°C for 1 day before the number of CFU was determined.

## RESULTS

The antibacterial activities of (*E*)-2-hexenal, indole, polymyxin B, and colistin against the two gram-negative bacteria are listed in Table 1. Since the MICs of (*E*)-2-hexenal and indole were not individually potent, further study was not warranted for their activity alone. Therefore, emphasis was placed

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TABLE 1. MICs and MBCs of the phytochemicals and polymyxins[a]

| Microorganism | (*E*)-2-Hexenal | | Indole | | Polymyxin B | | Colistin | |
|---|---|---|---|---|---|---|---|---|
| | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC |
| *P. aeruginosa* | 400 | 400 | 800 | 800 | 6.25 | 6.25 | 200 | 200 |
| *E. coli* | 400 | 400 | 800 | 800 | 3.13 | 3.13 | 50 | 50 |

[a] Results are expressed as micrograms per milliliter.

on evaluating their possible role as potentiators for the more active polymyxins.

The results from checkerboard combination assays are presented in Tables 2 and 3. FIC indices and the fold increases in activity levels were calculated, and the concentrations at which the highest levels of activity were exhibited are listed. The FIC index best identifies the combinations in which all compounds involved have the highest levels of activity at the lowest concentrations. It therefore has a limitation in that a low number requires the lowest concentrations of all compounds involved. Phytochemicals often exhibit synergy at higher concentrations, often at one-half of the MIC (9–14, 17). This is also true for potentiation of polymyxin activity. However, the use of one-half of the MIC automatically results in an FIC index of 0.5 regardless of the potentiation effect, and the combination will be considered only additive according to strict interpretation of the index value. An independent value that also characterizes the level of activity is the fold-increase value, which is the quantitative decrease in the MIC for one of the compounds identified alone versus the MIC for that compound in combination. For the data presented in Tables 2 and 3, the FIC indices and fold-increase values are calculated from the same MIC data derived from the checkerboard matrix and are therefore correlated. The fold increases in antimicrobial activity were presented along with FIC indices to give a more accurate picture of the effects of compound combinations.

Polymyxin B was less susceptible to potentiation effects than was colistin. In the two-compound combination study, additions of (*E*)-2-hexenal or indole to polymyxin B resulted in an FIC index of 0.625 for each of the two organisms, equivalent to an eightfold increase, indicating only an additive effect. A time-kill curve assay was done to more fully characterize the activity, which showed that 1.56 μg of polymyxin B per ml combined with 400 μg of indole per ml was bactericidal for *P. aeruginosa* (Fig. 1). The combination of all three compounds resulted in synergistic activity, with an FIC index of 0.687 for each microorganism, equivalent to a 16-fold increase. A time-kill curve for *E. coli* is presented in Fig. 2, which shows that the

TABLE 2. MICs, FIC indices, and fold-increase values for two- and three-compound combinations with polymyxin B sulfate[a]

| Microorganism | MIC (μg/ml) of: | | | FIC index | Fold increase[b] |
|---|---|---|---|---|---|
| | Polymyxin B | (*E*)-2-Hexenal | Indole | | |
| *P. aeruginosa* | 3.13 | 200 | | 1.001 | 2 |
| | 0.78 | | 400 | 0.625 | 8 |
| | 0.39 | 50 | 400 | 0.687 | 16 |
| *E. coli* | 1.56 | | 400 | 0.998 | 2 |
| | 0.39 | 200 | | 0.625 | 8 |
| | 0.195 | 200 | 100 | 0.687 | 16 |

[a] Concentrations exhibiting the highest level of activity are reported from checkerboard assays.
[b] Comparison of MICs of polymyxin B in combination versus alone.

TABLE 3. MICs, FIC indices, and fold-increase values for two- and three-compound combinations with colistin methanesulfonate[a]

| Microorganism | MIC (μg/ml) of: | | | FIC index | Fold increase[b] |
|---|---|---|---|---|---|
| | Colistin | (*E*)-2-Hexenal | Indole | | |
| *P. aeruginosa* | 50 | 100 | | 0.500 | 4 |
| | 50 | | 200 | 0.500 | 4 |
| | 6.25 | 200 | | 0.531 | 32 |
| | 1.56 | | 400 | 0.508 | 128 |
| | 25 | 100 | 50 | 0.438 | 8 |
| | 0.78 | 200 | 100 | 0.629 | 256 |
| | 0.39 | 50 | 400 | 0.627 | 512 |
| *E. coli* | 6.25 | 200 | | 0.625 | 8 |
| | 6.25 | | 400 | 0.625 | 8 |
| | 1.56 | 200 | 100 | 0.656 | 32 |

[a] Concentrations exhibiting the highest level of activity are reported from checkerboard assays.
[b] Comparison of MICs of colistin in combination versus alone.

MBC for polymyxin B in the three-compound combination is the same as the MIC, 0.195 μg/ml. In combinations with polymyxin B, indole was more active against *P. aeruginosa*, while (*E*)-2-hexenal was more active against *E. coli.*

Colistin was surprisingly susceptible to potentiation effects, although results differed between the microorganisms (Table 3). Assays with *E. coli* showed only an additive effect for the two-compound combination but moderate synergy for the three-compound combination, with 8- and 32-fold increases in colistin activity, respectively. *P. aeruginosa* was the most susceptible to potentiation for two-compound and three-compound combinations. In the double combinations, FIC indices indicate borderline synergy, but relatively equivalent values of 0.5 through 0.531 have greatly different fold-increase values. Higher concentrations of the phytochemicals gave increased potentiation effects. The addition of one-half of the MICs of indole and (*E*)-2-hexenal resulted in the highest degree of potentiation of colistin activity, 128- and 32-fold increases, respectively. Three-compound combinations were all synergistic, but FIC indices and fold-increase values differed as well. An FIC index of 0.438 corresponded to an 8-fold reduction in the MIC of colistin, while an FIC index of 0.627 corresponded to a 512-fold MIC reduction. One-half of the MIC of (*E*)-2-

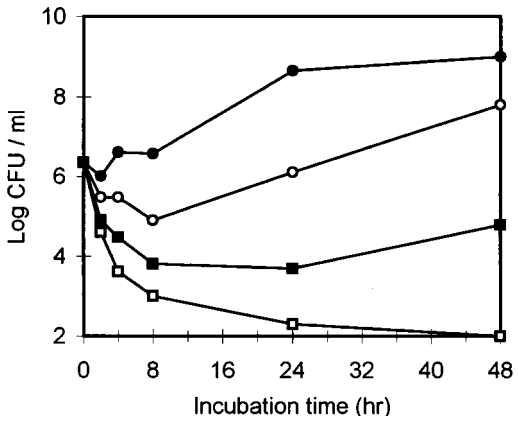


FIG. 1. Two-compound combination effects of polymyxin B with indole against *P. aeruginosa*. A 48-h culture was inoculated into NYG broth containing 400 μg of indole per ml (one-half MIC) in combination with 0.39 (○), 0.78 (■), and 1.56 (□) μg of polymyxin B per ml. ●, compound-free control.

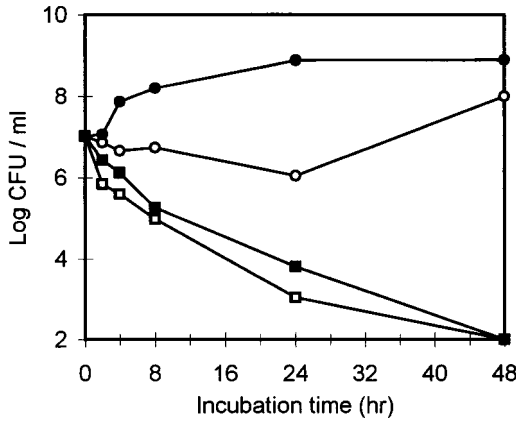


FIG. 2. Three-compound combination effects of polymyxin B with (*E*)-2-hexenal and indole against *E. coli*. The number of viable cells was determined in NYG broth containing, per milliliter, 200 μg of (*E*)-2-hexenal (one-half MIC), 100 μg of indole (one-eighth MIC), and 0.098 (○), 0.195 (■), and 0.39 (□) μg of polymyxin B. ●, compound-free control.

hexenal combined with one-eighth of the MIC of indole resulted in a 256-fold potentiation of colistin activity, while one-half of the MIC of indole combined with one-eighth of the MIC of (*E*)-2-hexenal increased colistin antimicrobial activity 512-fold. Figure 3 shows a time-kill curve for the 512-fold increase for the three-compound combination.

## DISCUSSION

Phytochemicals usually exhibit less antimicrobial activity than agents derived from microbial sources. This is likely due to the multichemical approach of a plant's defense, which utilizes various compounds with moderate antimicrobial activity. Thus, phytochemicals have often been overlooked as agents for antimicrobial therapy.

Polymyxins exhibit a lack of absorption when administered topically to the skin or orally in the digestive tract and thus do not diffuse through body membranes and tissues. Because of these properties they are not effective in deep-seated infections of tissues. However, they are effective in the treatment of infections limited to surfaces such as the skin, intestine, or eye.

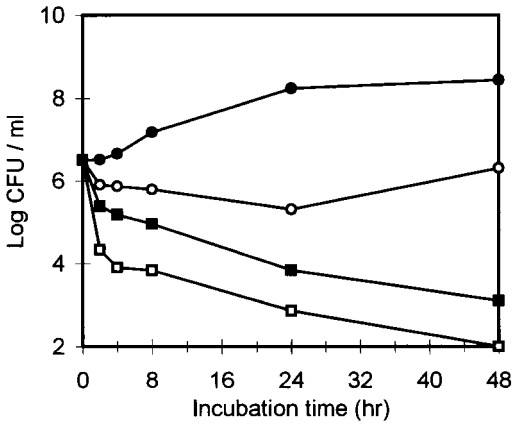


FIG. 3. Three-compound combination effects of colistin with (*E*)-2-hexenal and indole against *P. aeruginosa*. The number of viable cells was determined in NYG broth containing, per milliliter, 400 μg of indole (one-half MIC), 50 μg of (*E*)-2-hexenal (one-eighth MIC), and 0.195 (○), 0.39 (■), and 0.78 (□) μg of colistin. ●, compound-free control.

Since indole and (*E*)-2-hexenal both have broad spectrums of antimicrobial activity (12, 16), their combination with polymyxins could broaden the antimicrobial spectra of therapeutic combinations. This combination would work well for applications such as burn wounds and skin grafts. In fact, only recently has the clinical efficacy of the traditional polymyxin B, bacitracin, and neomycin combination been tested (2). The results verified the clinical potency against gram-positive and gram-negative bacteria. However, neomycin has been implicated in contact sensitization and may warrant a substitution. In a sensitization test of indole, a 1% concentration in petrolatum reportedly produced no sensitization reactions (6), which makes indole a potential candidate.

The enhancement of the activities of both polymyxin B and colistin when each was tested in combination with both of the phytochemicals suggests that the polymyxins, (*E*)-2-hexenal, and indole have different modes of actions. Since the compounds were combined in sublethal amounts, the activity observed must be due to the combination effect of the three compounds. This deduction is quantitatively supported in that the triple-combination FIC indices against both bacteria are significantly less than 1, indicating synergy. Indole and (*E*)-2-hexenal together seem to exhibit higher levels of activity than they do separately, as indicated by the differences between double- and triple-combination FIC indices and fold-increase values.

The mechanism of action of the polycationic polymyxins involves interaction with the outer membrane and destabilization of its structure via displacement of divalent cations from lipopolysaccharide and perhaps lipid bilayer disruption as well (8). The modes of action of (*E*)-2-hexenal and indole are currently being investigated.

The results presented here indicate the potential efficacy of phytochemical combinations with antibiotics to enhance total biological activity. By enhancing the activities of polymyxin B and colistin with phytochemicals, the amount of antibiotic used can be reduced, thus lowering its toxicity, or the amount of antibiotic can be maintained, thus delivering a more potent medicine. Not only can combinations increase activity, they can also deter the development of resistance. Indole and (*E*)-2-hexenal are attractive because they occur naturally in food and other plants and are currently approved by the Food and Drug Administration as food additives (Code of Federal Regulations 21 172.515) (4). Although low concentrations of compounds are usually desired for inclusion in medications, this condition is not exclusive. Since toxicity varies on the basis of the inherent properties of the compound and its method of administration, high concentrations may be efficacious in certain applications, a prime example of which is topical applications.

The potentiation of colistin activity is particularly noteworthy. It has not been commonly used because of its low levels of activity, but these results indicate a potency equal to that of polymyxin B when colistin is used in combination with these phytochemicals. It may therefore be worthy of consideration as a substitute for polymyxin B under some circumstances.

This is our first report of synergistic activity against gram-negative bacteria by phytochemicals, since phytochemicals exhibiting activity against this type of bacteria are rare. However, they may prove to be increasingly valuable as potentiators of more-active antibiotics. More importantly, they may provide another means of studying the mechanisms of bacterial control at a molecular level, since phytochemicals are structurally different from antibiotics and often have different modes of actions.

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