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Discovery and Development of Sesquiterpenoid Derived Hydroxymethylacylfulvene: A New Anticancer Drug

Trevor C. McMorris*

Department of Chemistry and Biochemistry, University of California, San Diego, La Jolla, CA 92093, USA

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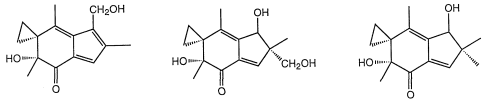
Abstract—Hydroxymethylacylfulvene (HMAF, MGI 114) is derived from the sesquiterpene illudin S by treatment with dilute sulfuric acid and excess paraformaldehyde. It is less cytotoxic than illudin S but exhibits much greater selectivity in toxicity to malignant cells compared to normal cells. HMAF is believed to undergo bioreductive activation in vivo. It is now being tested in human clinical phase II trials against solid tumors. © 1999 Elsevier Science Ltd. All rights reserved.

Introduction

Hydroxymethylacylfulvene (HMAF, also MGI 114, **1**) is a very promising antitumor compound derived from the sesquiterpene illudin S (**2**).[1] The latter was first isolated many years ago by Anchel et al., at the New York Botanical Garden during a search for antibiotic substances from Basidiomycetes.[2] Illudin S had high activity against *Staphylococcus aureus* but was extremely toxic to animals. In tests carried out by the National Cancer Institute, illudin S and illudin M (**3**) were found to have an unfavorable toxicity profile. During the past 10 years extensive preclinical studies on HMAF and related fulvenes have been carried out leading to phase I human clinical trials which began in December 1995.[3] Phase II clinical trials targeting several solid tumor types are now in progress under the sponsorship of MGI Pharma, a pharmaceutical company, and the National Cancer Institute.[4] This review recounts the discovery and development of these novel antitumor drugs.



1 2 3

Early Work on Illudins

The jack o'lantern mushroom *Omphalotus illudens* (formerly *Clitocybe illudens*), so called because of its bioluminescent property, is found in clusters at the base of trees, or on buried wood, in many areas of the United States and Canada.[5] Related species occur in Europe and Japan. Illudin S has been isolated from the mushroom *Lampteromyces japonicus* growing in Japan and shown to be the antitumor and toxic principle.[6,7] Much greater yields of illudin S can be obtained from liquid cultures of the fungus.[2] Several other metabolites including illudin M (**3**), illudol (**4**),[8] illudalic acid (**5**) and illudinine (**6**)[9] are produced as well. Many related sesquiterpenes have been isolated from Basidiomycetes.[10] Among them are illudin A and B (**7**, **8**), illudalenol (**9**)[11] and leaianafulvene (**10**).[12]

The structures of illudin S and illudin M were first reported by McMorris and Anchel in 1963.[13] Subsequently, two different Japanese groups independently verified the structural assignment of illudin S.[6,7] An X-ray crystallographic analysis of illudin S has also been carried out.[14]

Several proposals concerning the biosynthesis of illudins have been made. These sesquiterpenes are derived from farnesyl pyrophosphate via a tricyclic intermediate possessing a cyclobutane ring, i.e. a protoilludane skeleton[15,16] (Scheme 1). In fact, the metabolite illudol (**4**) was found to possess this kind of ring system. Many families of sesquiterpenes are now known to be biosynthesized from a protoilludane intermediate.[10] Another remarkable development has been the discovery of closely related sesquiterpenes in bracken fern (*Pteridium aquilinum*) and other ferns. A toxic principle in bracken was shown to be the nor-sesquiterpene glucoside, ptaquiloside (**11**).[17] This unstable glucoside readily breaks down giving the aromatic indanone pterosin B (**12**), one of a very large number of pterosins found in various ferns and fungi.[18]

*Tel.: +1 619 534-4178; fax: +1 619 534-4864.

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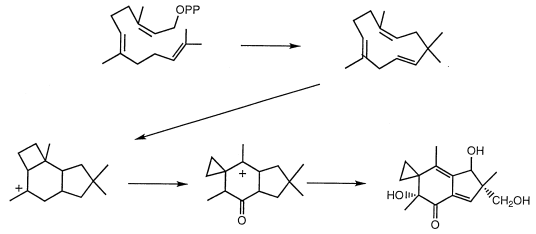
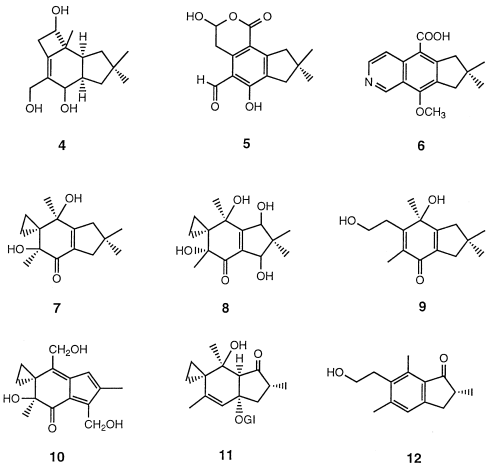
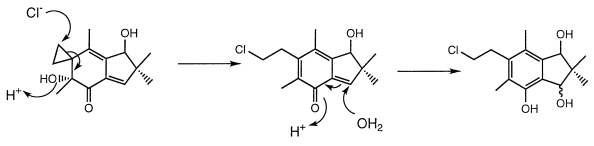


Scheme 1.

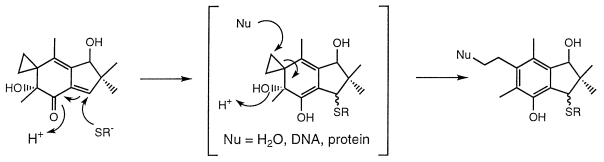


Studies on the Mechanism of Action of Illudins

The occurrence of similar toxic compounds, ptaquiloside and illudin S, in unrelated organisms, bracken fern and jack o'lantern mushrooms, was most intriguing and led me to investigate the mechanism of toxicity of these compounds. I felt that clarification of this mechanism might enable us to design analogues which would be less toxic but still retain antitumor properties. The structures of illudin S and M suggested that they could act as alkylating agents. At low pH (dilute HCl) illudin M reacts as shown in Scheme 2. Loss of the tertiary hydroxyl occurs together with opening of the cyclopropane ring and chloride ion acts as the nucleophile. The intermediate formed in this reaction is a quinone methide which reacts rapidly with water to give the stable aromatic product.[14] At neutral pH illudins are unreactive to oxygen, nitrogen or halogen nucleophiles. However, thiols react readily at room temperature, adding to the α,β-unsaturated carbonyl. The cyclohexadiene intermediate rapidly undergoes opening of the cyclopropane and loss of the tertiary hydroxyl (Scheme 3). The overall result is addition of two nucleophiles.[19]



Scheme 2.



Scheme 3.

Reaction with thiols (e.g. methylthioglycolate, cysteine and glutathione), is pH-dependent, the optimum pH being 5.6–6.1. Not surprisingly, toxicity of illudins can be modulated by varying glutathione levels in cells. Experiments with human leukemia (HL 60) cells pretreated with agents that depress or enhance glutathione levels confirmed that illudins were more toxic to cells with depressed glutathione levels and less toxic to cells with enhanced glutathione levels.[19]

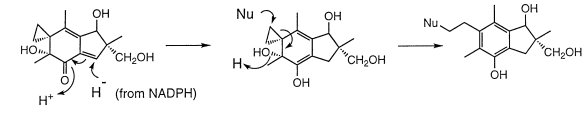
The toxicity of illudins might therefore be a result of spontaneous reaction with enzymes containing thiol groups, e.g. glyceraldehyde 3-phosphate dehydrogenase or ribonucleotide diphosphate reductase. There is also the possibility that alkylation of DNA or protein by the cyclohexadiene intermediate from reaction with glutathione contributes to the toxicity.

Illudin S can be converted to an aromatic product with NADPH and rat liver cytosol.[20] Presumably addition of hydride to the α,β-unsaturated ketone gives the activated intermediate, similar to the one in the thiol reaction, triggering opening of the cyclopropane ring. Alkylation of water or macromolecules (DNA, protein) thus occurs (Scheme 4). It is interesting to note that reductive reactions of illudins (e.g. with H_2/Pd) give aromatic products which were important in the structure elucidation of these sesquiterpenes.[21]

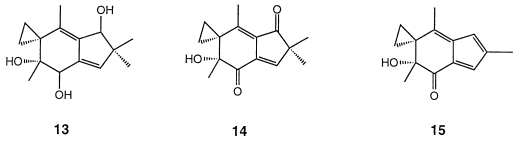
In our initial studies of the mechanism of toxicity of the illudins, dihydroilludin M (**13**) was prepared by reduction of illudin M with sodium borohydride. When tested in mice dihydroilludin M was found to be several orders of magnitude less toxic than the parent compound. This loss in toxicity was later confirmed by Kelner et al., who carried out cytotoxicity studies with human leukemia (HL60) cells. Illudin S and illudin M had similar IC_{50} values of 3 ± 1 nM while the IC_{50} value of dihydroilludin S or dihydroilludin M was greater than 10^4 nM. These results confirmed the importance of the α,β-unsaturated ketone moiety in toxicity of illudins.[22]

Development of New Antitumor Agents

Investigations of analogues of illudins led to new compounds with greater selectivity in their toxicity toward cancer cells compared to normal cells. The first of these



Scheme 4.



13 14 15

was dehydroilludin M (**14**), prepared by oxidation of illudin M with pyridinium dichromate. This compound was two orders of magnitude less toxic (IC_{50}~296 nM) than the parent illudin M (IC_{50}~3 nM) when tested on HL60 cells.[23] Dehydroilludin M reacted more slowly than illudin M with dilute HCl and with thiols. Thus toxicity correlated with reactivity to thiols.

Dehydroilludin M was found to have remarkable antitumor activity when tested on metastatic human lung cancer cells (MV522) implanted in nude mice.[24] It inhibited xenograft growth and prolonged life span of tumor bearing animals. The efficacy exceeded that of nine known anticancer drugs and equalled that of mitomycin C. Derivatives of illudin S were also examined. When dissolved in dilute acid (e.g. H_2SO_4) illudin S undergoes reverse Prins reaction with loss of formaldehyde yielding a fulvene, designated acylfulvene (**15**).[25,26] This compound possesses the key features, α,β-unsaturated ketone and cyclopropylmethyl carbinol, required to trigger alkylating action. In fact, the compound was found to be slightly less toxic to HL60 cells than dehydroilludin M (IC_{50} 415 ± 31 nM versus 296 ± 8 nM). It reacted much less rapidly than illudin S (about one sixtieth) with thiol. This decrease in reactivity can be explained by the resonance structures shown (Scheme 5).

Acylfulvene demonstrated marked efficacy against the MV522 xenograft, inhibiting both primary tumor growth and markedly increasing median life span.[27] Its efficacy exceeded that of dehydroilludin M, cisplatin, paclitaxel, and mitomycin C. Many acylfulvenes were then prepared and tested including the bisacylfulvene (**16**). None was more effective than acylfulvene except for HMAF.

Bisacylfulvene (**16**) is formed in a remarkable reaction when illudin S is dissolved in dilute H_2SO_4 (Scheme 6). Acylfulvene (**15**) and HMAF are intermediates in this reaction. The latter compound is highly reactive and was observed only in trace amounts. However, by carrying out the reaction under carefully controlled conditions, with a large excess of paraformaldehyde, a good yield of **1** was obtained.[1] We expected this compound to be more water-soluble than acylfulvene and therefore more suitable for biological tests. Also, the allylic hydroxyl appeared to be easily displaced albeit under strongly acidic conditions. Thus an additional site was present in the molecule at which nucleophilic attack could occur.

Scheme 5.

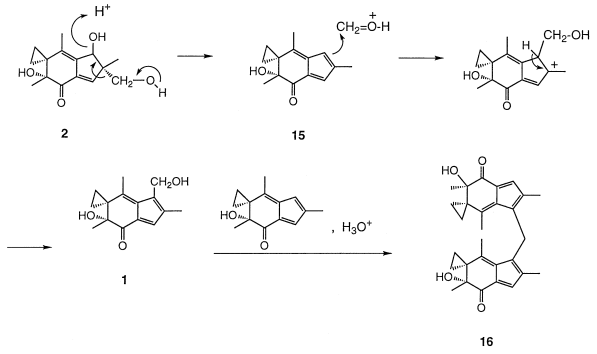
Preclinical Studies of HMAF

HMAF was first tested by Kelner et al., in the MV522 metastasizing lung carcinoma xenograft model.[28] HMAF induced primary tumor regression in all animals and increased life span more than 150%. In this model, treatment with paclitaxel, doxorubicin or *cis*-platin failed to significantly inhibit primary tumor growth or prolong life span. Mitomycin C at the LD20 increased life span by 61%. Equally impressive results were obtained with HMAF in other laboratories. For example, in a recent report[3] HMAF was found to be very effective in human tumor xenograft models, including MX-1 breast carcinoma, MV522 lung adenocarcinoma and HT-29 colon carcinoma, but not murine B-16 melanoma or P388 leukemia. Excellent responses were observed in animals bearing MX-1 tumors administered (iv or ip) doses of 3–7.5 mg/kg daily for 5 days, with complete regression recorded in 29 out of 30 animals. Extensive tumor shrinkage was also observed with MV522 and significant tumor growth inhibition was obtained with HT-29. Complete regressions were also observed in individual animals with MV522 and HT-29 tumors.

In a recent animal study where DU-145 human prostate tumors were implanted in mice, 5 out of 8 animals showed complete elimination of tumors. In a second study using PC-3 human prostate tumors, all 10 mice showed a partial shrinkage of their tumors (> 50% shrinkage) with a mean shrinkage of 72.5%. HMAF is active against many other solid tumor cell lines including ovarian, gastric, squamous cell and pancreatic.[29]

Another recent finding is the synergistic effect of HMAF in combination with irinotecan (ITN, a derivative of camptothecin).[29] When administered to mice bearing the HT-29 human colon xenograft there were several complete responses (with HMAF + ITN). However, with ITN alone there was tumor growth inhibition but no tumor shrinkage.

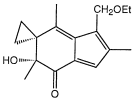
Because of its very promising antitumor activity HMAF was selected for extensive preclinical investigations with the objective of advancing this compound to phase I and phase II clinical trials. However, chemical investigation of HMAF and new analogues continues. For example, under acidic conditions HMAF reacts readily



2 15 1 16

Scheme 6.

with thiols and oxygen nucleophiles.[30] Products are formed in which the primary allylic hydroxyl has been displaced by sulfur or oxygen containing groups. The acylfulvene structure is still intact and, not surprisingly, these derivatives retain the antitumor activity of HMAF. Several analogues (e.g. **17**, **18**, **19**, **20**) have been tested in the NCI 60 tumor cell line in vitro screening assay. They demonstrated significant inhibitory effects on tumor cell growth in a wide variety of solid tumors. Non-small cell lung, ovarian, and renal tumor cell lines were generally more sensitive than other solid tumor types tested.



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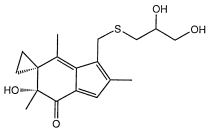

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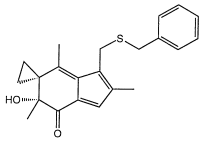

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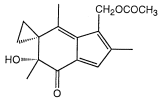

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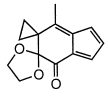
Total Synthesis of HMAF

HMAF being used in clinical trials is prepared semi-synthetically. The starting material is illudin S which can be obtained in high yield by fermentation of *Omphalotus illudens*, and converted to HMAF in dilute H_2SO_4 with paraformaldehyde. Illudin S and illudin M have been synthesized by Matsumoto et al.[31,32] but these syntheses are lengthy and not suitable for large scale production. Several investigations have been reported recently on the synthesis of illudin M.[33–35] In particular, Padwa et al. have described a 1,3-dipolar cycloaddition reaction which is very useful for constructing the illudin skeleton.[33] Padwa et al. and Kinder et al.[34] have independently synthesized illudin M, dehydroilludin M and related bicyclic analogues.[35] We have adapted Padwa's procedure for the synthesis of hydroxymethylacylfulvene (Scheme 7) and two analogues (**21**, **22**) which possess good antitumor activity.[36,37] Synthesis of ^{14}C HMAF has been accomplished by reaction of acylfulvene with ^{14}C-paraformaldehyde.[38]



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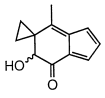

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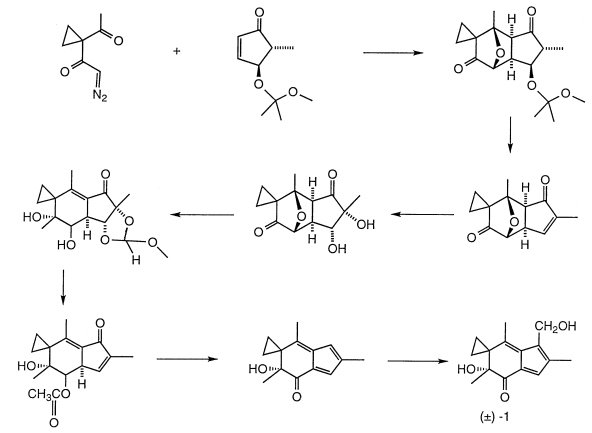
Metabolism of illudins and acylfulvenes

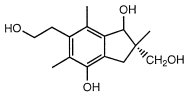
The extreme toxicity of illudins has been well known for sometime although studies of the metabolism of illudin S have been reported only recently. Tanaka et al. isolated two aromatic compounds (**23** and **24**) from incubation



Scheme 7.

of illudin S with a rat liver supernatant and NADPH.[20] These metabolites are the result of enzyme catalyzed reduction of illudin S by NADPH. The presumed cyclohexadiene intermediate behaves as a potent alkylating agent and reacts with water, chloride and also macromolecules. The enzyme responsible for this reaction appears to differ from known cytosolic enzymes implicated in xenobiotic metabolism.

Tanaka et al. have further demonstrated that metabolite (**23**) is excreted in the urine of rats after oral administration of illudin S.[39] It was found partly as the glucuronide. Stereoisomeric mercapturic acids (**25** and **26**) were also detected in the urine. These may have been formed via glutathione adducts with illudin S. We have confirmed that metabolism of illudin S by rat liver cytosol and NADPH yields two aromatic compounds (**23** and **24**) obtained by Tanaka et al. Metabolism of acylfulvene was examined and found to be much slower than that of illudin S.[40] Two metabolites were isolated (**27** and **28**). The former corresponds to compound **23** from illudin S and indicates a similar activation of the toxin. The other metabolite (**28**) was unexpected. It retains the toxicity and antitumor activity of acylfulvene and is metabolized further by rat liver cytosol yielding the aromatic compound **29**.



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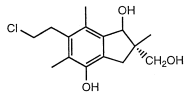

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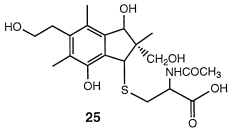

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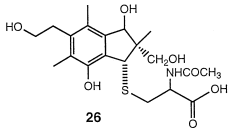

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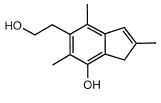

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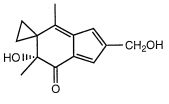

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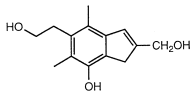

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Biological aspects of toxicity of illudins and acylfulvenes

Chemical studies on illudins and acylfulvene clearly indicate that these compounds function as alkylating agents. The superior antitumor properties of acylfulvenes may be a reflection of more selective alkylating ability. However, there are other aspects to the mechanism of action of these compounds that may be relevant. Kelner et al., have found that while illudins are toxic to most tumor cells after prolonged exposure ($\geq$48 h), with shorter exposure times ($\leq$2 h), they show selective toxicity for human myelocytic leukemia and epidermoid, lung, ovarian and breast carcinoma cells. The apparent histologic specificity of illudin S toxicity is based on an energy dependent transport mechanism present in sensitive cells, but absent in cells relatively resistant to illudin S.[41] Another important finding was that multidrug resistant (mdr) cells were just as sensitive to illudins as the parent cell lines.[42]

Selective toxicity to various cancer cells was retained in dehydroilludin M, acylfulvene and HMAF. The energy dependent transport mechanism was shown to be operating with acylfulvene and HMAF.

Illudins are believed to alkylate DNA after activation in the cell. The resulting DNA damage differs from that from other known DNA damaging toxins.[43] The sensitivity pattern of various UV-sensitive Chinese hamster ovary cell lines differs from that of known DNA crosslinking agents. Normally, the ERCC1 (excision repair cross complementing) and ERCC4 deficient cell lines are most sensitive to DNA-crosslinking agents, with ERCC2-, ERCC3- and ERCC5-deficient cell lines having minimal sensitivity. With illudins the pattern is reversed, ERCC2 and ERCC3 being the most sensitive. This sensitivity to illudins is attributed to a deficiency of ERCC2 and ERCC3 gene products. The finding that drug-resistant tumors display increased ERCC1, but not ERCC2, expression may explain illudin S efficacy against these tumors.

Woynarowski et al. have reported effects of HMAF on DNA integrity and apoptosis induction in CEM human leukemia cells.[44] No bifunctional lesions, interstrand DNA cross-links or DNA-protein cross-links were seen (by alkaline sedimentation and K^+/SDS precipitation, respectively) when using up to 50 μM HMAF. The drug possibly formed some monoadducts, as DNA from drug-treated cells impeded primer extension by Taq polymerase, although only partial inhibition was seen even at 200 μM HMAF. HMAF also induced secondary lesions in cellular DNA, single-strand breaks that were detectable after a 4 h treatment at HMAF levels as low as 2 μM, comparable to the growth inhibition IC_{50} value (1.7 μM). A post-treatment incubation of cells in drug-free medium generated substantial amounts of DNA double-stranded fragments of several kbp, suggesting apoptotic fragmentation. HMAF preferentially inhibited DNA synthesis ($IC_{50}\sim$2 μM), consistent with an S phase block, observed by cell cycle analysis.

Phase I and Phase II clinical trials

MGI Pharma acquired the rights to develop acylfulvenes as anticancer drugs from the University of California in 1993. This company has sponsored preclinical investigations and also Phase I trials which were designed to identify the highest dose of MGI 114 that can be safely administered to adults with solid tumors. Current results (as of August 1998) indicate that significant doses of the drug can be administered to humans before a dose-limiting degree of bone narrow suppression is observed. Other drug-related toxicities reported include nausea, vomiting, fatigue, facial flushing, phlebitis and reversible kidney effects.

MGI Pharma has initiated its Phase II clinical program with the treatment of hormone-refractory prostate cancer patients. The company plans to begin Phase II studies in ovarian and pancreatic cancer patients this year. The National Cancer Institute has announced the start of its Phase II trials which will target renal, colorectal, lung, breast and ovarian cancer.[4]

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