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Characterization of MGI 114 (HMAF) histiospecific toxicity in human tumor cell lines

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Abstract *Purpose*: The acylfulvenes are a class of antitumor agents derived from the fungal toxin illudin S. One acylfulvene derivative, MGI 114 (HMAF), demonstrates marked efficacy in xenograft carcinoma models when compared to the parent acylfulvene or related illudin compounds. The maximum tolerated dose (MTD) of the two analogs in animals, however, is similar. To help elucidate the basis of the increased therapeutic efficacy of MGI 114, we determined the in vitro cytotoxicity, cellular accumulation and DNA incorporation of this drug and compared the results with those from the parent acylfulvene analog. *Methods*: The cytotoxicity of acylfulvene analogs was tested in vitro against a variety of tumor cell lines. Radiolabeled MGI 114 was used for cellular accumulation and DNA incorporation studies. *Results*: MGI 114 retained relative histiospecific toxicity towards myeloid leukemia and various carcinoma cell lines previously noted with the parent acylfulvene compound. Markedly fewer intracellular molecules of MGI 114 were required to kill human tumor cells in vitro as compared to the parent acylfulvene, indicating that MGI 114 was markedly more toxic on a cellular level. At equitoxic concentrations, however, the incorporation of MGI 114 into genomic tumor cell DNA was equivalent to that of acylfulvene. Analysis of cellular accumulation of MGI 114 into tumor cells revealed a lower Vmax for tumor cells, and a markedly lower Vd for diffusion accumulation as compared to acylfulvene. *Conclusions*: The addition of a single methylhydroxyl group to acylfulvene to produce MGI 114 results in a marked increase in cytotoxicity in vitro towards tumor cells as demonstrated by the reduction in IC_{50} values. There was a corresponding decrease in the number of intracellular molecules of MGI 114 required to kill tumor cells, but no quantitative alteration in covalent binding of the drugs to DNA at equitoxic concentrations. This indicates that cellular metabolism plays a role in the in vitro cytotoxicity of MGI 114. The equivalent incorporation into genomic DNA at equitoxic doses suggests that DNA damage produced by acylfulvene and MGI 114 is equivalent in regard to cellular toxicity and ability to repair DNA. This increased cellular toxicity, together with the decrease in diffusion rate, may explain the increased therapeutic efficacy of MGI 114 as compared to the parent acylfulvene analog.

Key words Illudins · Acylfulvene · Chemotherapeutic · MGI 114 · HMAF

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Introduction

Illudins are sesquiterpene compounds derived from the mushroom *Omphalotus illudens* and related species of basidiomycetes [1–6], whose chemical structure differs from known chemotherapeutic agents [4]. The illudins are preferentially cytotoxic in vitro with short exposure periods (< 2 h) towards a variety of hematopoietic and solid tumor cells at nano- to picomolar concentrations [7, 8]. In contrast, normal bone marrow progenitors or fibroblasts are relatively resistant to illudins and require micromolar concentrations for equivalent in vitro cytotoxicity [7, 8]. This preferential illudin cytotoxicity may arise from differences in intracellular drug accumulation

due to variation in expression of an energy-dependent uptake process [8]. In contrast to DNA damage produced by many other anticancer drugs, repair of illudin-induced DNA damage appears to require early action of the ERCC2 and ERCC3 DNA helicases before repair proceeds [9].

Although illudin S (Fig. 1) has proved too toxic for effective use in vivo [7, 10], we have synthesized analogs with a dramatically improved therapeutic index in vivo against experimental tumors compared to the parent compound. The first group (dehydroilludins) increase the lifespan of mice bearing human lung carcinoma xenografts resistant to ten conventional chemotherapeutic agents, including paclitaxel [10, 11]. The second group of analogs (acylfulvenes, Fig. 1) are even more effective in lung xenograft models [12]. The acylfulvene analog, MGI 114 (6-hydroxymethylacylfulvene or HMAF, Fig. 1), has demonstrated marked efficacy in the MV522 lung carcinoma, MX1 breast carcinoma, and HT29 colon carcinoma xenograft models [13, 14]. MGI 114 has completed phase I chemotherapeutic clinical trials and 12 phase II trials were initiated in 1998, with promising preliminary results.

The pharmacologic basis for the increased therapeutic efficacy of MGI 114, as compared to the parent acylfulvene analog, is unknown. The structural difference between the two agents consists of a single methylhydroxyl group (Fig. 1). The increased antitumor efficacy of MGI 114 may arise from variation in energy-dependent accumulation in tumor cells, intracellular metabolism, DNA damage or repair, in vivo pharmacokinetics, tissue distribution, or increased solubility. We present evidence that the increased efficacy of MGI 114 arises from its increased inherent drug toxicity towards cells and not from differences in energy-dependent cellular accumulation parameters or DNA binding.

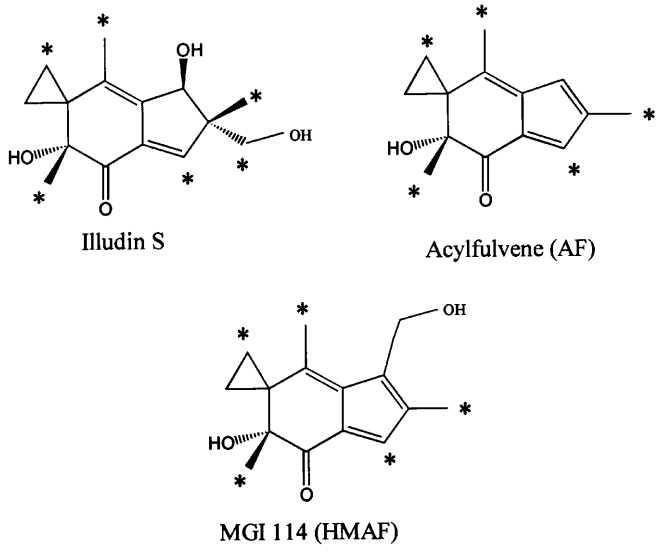

Fig. 1 Chemical structures of the illudin derivatives studied. The *asterisks* indicate the positions of the tritium atoms in the radiolabeled derivatives

Materials and methods

Cell lines

The following cell lines were maintained in either RPMI-1640 or Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Hyclone, Logan, Utah) as previously described [7, 8]: human myeloid leukemia cell line HL60 [15], human B-cell-derived leukemia/lymphoma cell line 8392 [16], human lung adenocarcinoma cell line MV522 [17], colon adenocarcinoma lines HT29 [18] and SW48 [19], and human breast carcinoma cell lines MCF7 [20] and MDA231 [21, 22]. All cell lines were routinely screened for mycoplasma. The cytotoxic effect of MGI 114 in vitro was determined by either cell count (trypan blue exclusion), inhibition of thymidine incorporation into DNA, or clonogenic colony forming assays (CFA) as previously described [7, 8].

Preparation of illudin analogs and radiolabeled acylfulvene

Omphalotus illudens subtype S.B. Carey 4435 (formerly *Clitocybe illudins*) was obtained from the New York Botanical Garden (New York, N.Y.). Illudin S (Fig. 1) was prepared from cultures as previously described [1–3]. The acylfulvene analog (Fig. 1) was prepared from the parent compound illudin S by reaction with 2 *M* H_2SO_4 [24, 25]. Radiolabeled illudin S was prepared by the addition to the fermentation broth of the precursor tritiated sodium acetate [9]. The radiolabeled illudin S was converted to radiolabeled acylfulvene as previously described [12], which was then converted to radiolabeled MGI 114 [33]. The specific activity of the tritiated MGI 114 used in this study was 310 mCi/mmol.

Illudin cellular and genomic DNA accumulation studies

Cellular uptake of tritiated MGI 114 into tumor cells was performed as previously described [26]. Briefly, the cells were incubated with radiolabeled drug in 10 ml total volume at 37 °C. After incubation the cells were washed with ice-cold saline three times, and collected. Cells were separated from the saline by a layering technique described previously [35] except 10% bovine serum albumin (BSA) was used. The saline suspension containing cells was carefully layered over the 10% BSA, the layers centrifuged for 3 min (IEC 428 centrifuge) at the lowest possible setting (60 *g*), and the centrifuge allowed to stop without braking. The majority of the radiolabeled drug remained in the upper layer while the cells were centrifuged into the lower layer. Aspiration at low vacuum was used to remove the upper layer first, and then the lower layer, leaving the cells behind. Residual moisture in the tube could be removed by touching the tip of a tissue to the side of the tube and the pellet without disturbing the cells. For determination of residual radioactivity the cells are suspended in Aquasol which simultaneously lyses the cell membranes. With this technique an experienced person can consistently achieve background counts (determined by adding ice-cold radioactive drug to ice-cold cells and immediately centrifuging) of only 50 to 60 cpm (Beckman LS 6800 scintillation counter, Irvine, Calif.), or $< 1\%$ of values obtained in the incubation studies. The incorporation of tritiated MGI-114 into cellular genomic DNA was performed as previously described using a G-NOME DNA Isolation Kit (BIO-101, Vista, Calif. [9]. The incorporation of tritiated MGI 114 into purified DNA was performed by incubating the drug with calf thymus DNA for 2 h at 37 °C in normal saline/phosphate buffer (pH 7.4). The DNA was precipitated by adding ethanol to a final concentration of 70%. The MGI 114 remained dissolved in the 70% ethanol solution. The DNA was collected by centrifugation at 4 °C for 15 min (15 000 *g*). The DNA pellet was washed three times with

Table 1 Cytotoxicity of MGI 114 in various human tumor cell lines. Values are the mean IC_{50} (n*M*) ± SD from three or four experiments (*Td* thymidine inhibition assay, *CFA* colony-forming assay, *TB* trypan blue exclusion assay)

Cell line	2-h Td	2-h CFA	48-h TB
HL60	150 ± 11	830 ± 90	170 ± 20
SW48	1900 ± 40	1200 ± 130	420 ± 50
HT29	250 ± 40	1060 ± 70	460 ± 60
MCF7	540 ± 80	160 ± 25	90 ± 30
MDA231	520 ± 80	350 ± 50	50 ± 4
MV522	110 ± 40	1200 ± 100	73 ± 8
8392	7350 ± 2100	26,000 ± 4500	76 ± 4

a 70% ethanol solution and suspended in water, and the residual radioactivity determined.

Statistics

Statistical analysis was performed using linear regression (least squares), correlation coefficients, and the nonparametric Mann-Whitney analysis for comparing MGI 114 to acylfulvene in the cytotoxicity, cellular accumulation, and the cellular genomic DNA studies. Analysis was performed using Graph Pad Instat Software, version 2.02 (La Jolla, Calif.).

Results

Cytotoxicity studies

Several cell lines had previously been shown to be comparatively sensitive to illudin S and acylfulvene after a 48-h exposure, but marked variation in cell line sensitivity was noted using a 2-h dosing exposure [7, 8, 12]. MGI 114 retained this pattern as all cells tested were relatively sensitive using a 48-h exposure (Table 1). Cell lines previously noted to display energy-dependent cellular accumulation of illudin S and acylfulvene [8, 30, 32] were also sensitive to MGI 114 using a 2-h exposure. These lines included the myeloid HL60, breast carcinoma MCF7, breast carcinoma MDA231, colon carcinoma SW48, colon carcinoma HT29, and lung carcinoma MV522. In contrast, the 8392 B-cell line, previously shown to lack energy-dependent accumulation of illudin S and acylfulvene [8, 12, 30, 32], was relatively resistant to MGI 114 after a short 2-h exposure (Table 1). The IC_{50} obtained using colony-forming assays (CFA) correlated ($r = 0.974$, $P < 0.01$) with values obtained using the rapid thymidine inhibition incorporation assay (Table 1). Although MGI 114 was more effective than either illudin S or acylfulvene in xenograft models [10, 12, 13, 31], the 2-h or 48-h IC_{50} values for this agent were intermediate between those of illudin S and acylfulvene for the cell lines tested (Fig. 2).

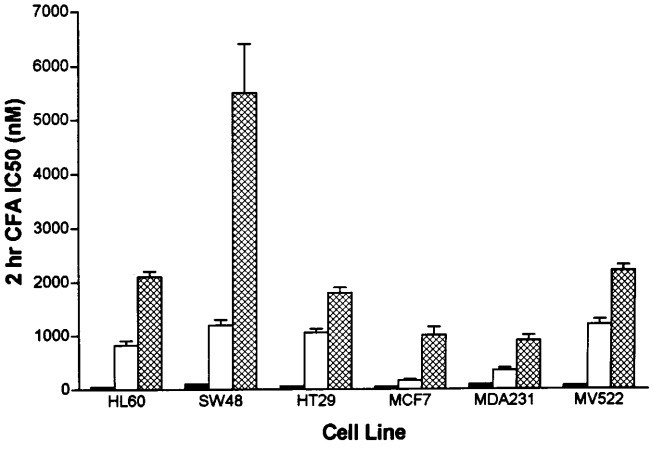

Fig. 2 Comparison of the cytotoxicities of MGI 114 (□), acylfulvene (▨) and illudin S (■) in human tumor cell lines. Values represent the means ± standard deviation from three experiments

Cellular uptake and incorporation into genomic DNA

Tumor cells were incubated with MGI 114 at the low concentration of 407 n*M* (100 ng/ml) and the cellular accumulation determined (Table 2). The number of intracellular MGI 114 molecules required to kill 50% of cells (determined by CFA) after a 2-h exposure was calculated to enable comparison with the results of previous studies using illudin S and acylfulvene. Despite marked variation in the in vitro IC_{50} (Table 1), the estimated number of MGI 114 molecules required to kill cells appeared to be relatively constant with the exception of the 8392 B cell lymphoma/leukemia cell line (Table 3). The number of MGI 114 molecules required to kill a specific tumor cell line, however, was significantly less (Mann-Whitney $P < 0.01$) than the number of parent acylfulvene molecules required (Table 3), but not significantly different from the number of illudin S molecules required ($P > 0.3$). The amount of MGI 114 incorporated into genomic DNA (at the respective IC_{50}) in the various cell lines (Table 4) was also not signifi-

Table 2 Cellular accumulation of MGI 114 into human tumor cell lines following a 2-h exposure to 407 n*M* (100 ng/ml). Values are means ± SD from three experiments

Cell line	2-h total (pmol/10 million cells)
HL60	9 ± 2
MV522	14 ± 2
SW48	10 ± 1
HT29	17 ± 1
MDA231	11 ± 1
MCF7	33 ± 5
8392	2 ± 1

Table 3 Number of molecules of MGI 114 required to kill 50% of cells with a 2-h exposure as determined by CFA. Acylfulvene and illudin S values are as previously reported [30, 32] and are provided here to allow direct comparison with MGI 114. Values are means ± SD from three experiments

Cell line	MGI 114 (molecules × 10^3 cell)	Acylfulvene (molecules × 10^3 cell)	Illudin S (molecules × 10^3 cell)
HL60	1700 ± 80	3000 ± 420	78 ± 12
MV522	1400 ± 6.5	29 700 ± 600	1720 ± 70
8392	17 400 ± 900	24 600 ± 4400	852 ± 108
SW48	1050 ± 35	13 900 ± 300	452 ± 30
HT29	2450 ± 100	39 200 ± 1100	480 ± 18
MDA231	580 ± 10	8700 ± 300	516 ± 18
MCF7	650 ± 60	7700 ± 800	1140 ± 36

Table 4 Binding of MGI-114 to cellular DNA or RNA after a 2-h exposure to the MGI 114 IC_{50} shown for each cell line as determined in the CFA. Values are means ± SD from three experiments

Cell line	IC_{50} (n*M*)	DNA incorporation (fmol/μg)	RNA incorporation (fmol/μg)
HL60	825	12.7 ± 2.5	1.1 ± 0.1
MV522	1200	22.7 ± 3.2	8.6 ± 0.6
MDA231	350	12.3 ± 1.13	
MCF7	160	7.3 ± 1.1	
SW48	1220	13.8 ± 1.7	
HT29	1060	28.0 ± 2.1	
8392	26 000	28.6 ± 0.3	

cantly different than the amounts of acylfulvene incorporated into genomic DNA (Fig. 3) at respective IC_{50} values (Mann-Whitney $P > 0.3$) [30, 32]. There was also no detectable binding of MGI 114 when purified genomic DNA was exposed to radiolabeled MGI 114 (100 ng/ml) for 2 h at 37 °C (data not shown).

Characterization of acylfulvene energy-dependent cellular accumulation

The cellular accumulation of MGI 114 into tumor cells with time was assessed. Similar to results previously obtained with illudin S and acylfulvene [12], the MGI 114 uptake into myeloid HL60 and into lung carcinoma MV522 cells increased with time and was retarded by the metabolic inhibitors, 2-deoxyglucose and antimycin A (Fig. 4). The time-dependent cellular accumulation of MGI 114 was markedly lower in the resistant 8392 B-cell line.

Energy-dependent uptake of MGI 114 into both MV522 and HL60 cells was concentration dependent at low external concentrations, but saturated at high concentrations (data not shown). The energy-dependent MGI 114 cellular uptake Vmax[1] and Km in HL60 myeloid and MV522 lung carcinoma cells were similar to the values previously noted for illudin S (Table 5). The Vmax of MGI 114, however, was lower than the value for the acylfulvene analog in MV522 cells (Table 5). We were unable to adequately assess energy-dependent cellular accumulation in the 8392 cells due to low cellular uptake of the drug. We were also unable to determine the diffusion coefficient (Vd) for MGI 114 due to the low cellular uptake at 4 °C (Table 5).

Discussion

MGI 114 (Fig. 1) has previously been noted to be highly effective in a metastatic xenograft lung carcinoma model resistant to conventional chemotherapeutic

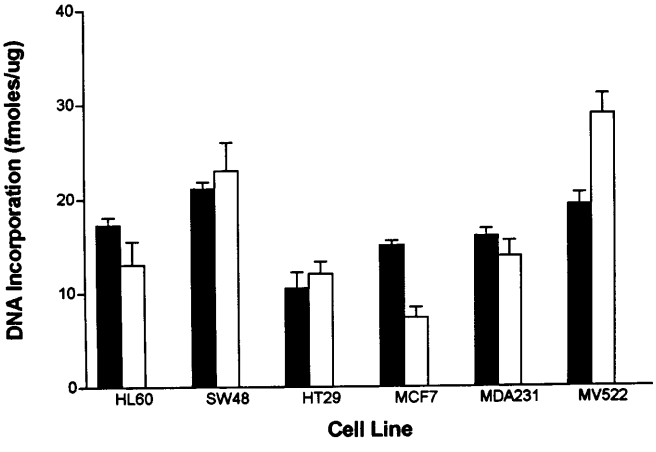

Fig. 3 Binding of MGI 114 (□) and the parent analog acylfulvene (■) to cellular genomic DNA in human tumor cell lines at the 2-h IC_{50} concentration (from colony-forming assays) for each compound. Values represent the means ± standard deviation from three experiments

[1] Linear transformation of the Michaelis-Menten equation does not weight all data points equally, and often leads to erroneous determination of kinetic parameters. The use of nonlinear regression algorithms which fit data directly to the equation, without prior weighting of data points, eliminates this problem. For further discussion see reference 29.

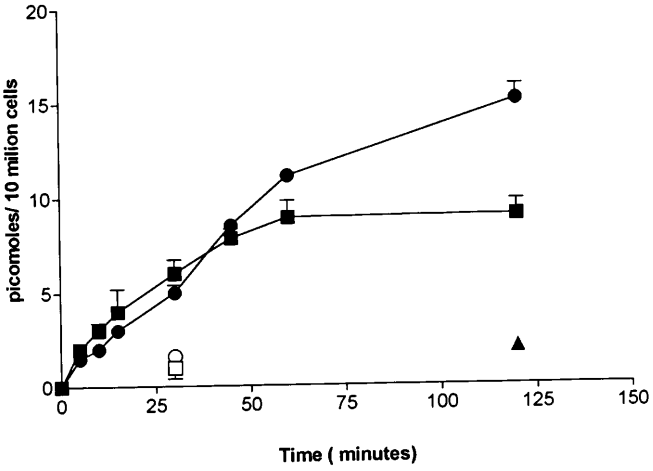

Fig. 4 Cellular accumulation of MGI 114 as a function of time in HL60 myeloid leukemia cells (■) and MV522 lung carcinoma cells (●). The isolated points represented by the *open square* and the *open circle* indicate MGI 114 uptake in the presence of the metabolic inhibitors 2-deoxyglucose and antimycin A into HL60 and MV522 cells, respectively. The isolated point represented by the triangle indicates MGI 114 uptake into 8392 B-cell lymphoma/leukemia cells at 2 h. Values represent the means ± standard deviation from three experiments

agents [12, 31]. The present studies demonstrate that MGI 114 retains selective in vitro cytotoxicity towards myeloid leukemia, breast, colon, and lung carcinoma cells using short (2-h) exposure periods (Fig. 2) exhibited by other illudins. In contrast, prolonged exposure allowed illudins to passively diffuse across cell membranes, while selective in vitro cytotoxicity required short exposure times and correlated with energy-dependent illudin intracellular accumulation [8, 30, 32]. With prolonged exposure (48 h), the toxicity of MGI 114 towards the 8392 B-cell line became equivalent to that noted with sensitive cell lines (Table 1).

Despite having similar in vivo MTD, MGI 114 was more toxic in vitro than acylfulvene, as demonstrated by the lower IC_{50} values in both the 2-h thymidine assay and the 2-h CFA. This increased in vitro killing of tumor cells by MGI 114 was accomplished with up to a 12-fold lower incorporation of radiolabeled equivalents of MGI 114 per cell as compared to acylfulvene (Table 3), indicating that MGI 114 is more toxic on a cellular level than the parent acylfulvene compound (nonparametric Mann-Whitney $P < 0.01$). At equitoxic concentrations (IC_{50}), the incorporation of MGI 114 into tumor cell genomic DNA (Table 4) was equivalent ($P > 0.20$) to incorporation of acylfulvene or illudin S [12, 30, 32]. The relatively low number of MGI 114 intracellular molecules required for cell killing, combined with the equivalent genomic DNA binding, indicates that cellular metabolism may be a factor in the increased in vitro cytotoxicity of MGI 114 as compared to that of acylfulvene analog. In agreement with previous studies on illudins and acylfulvene [7, 8, 30, 32], inability to detect spontaneous binding of radiolabeled MGI 114 to purified DNA indicates that intracellular metabolism to a DNA-reactive intermediate is required. Analysis of cellular accumulation of MGI 114 into MV522 tumor cells revealed a lower Vmax for energy-dependent accumulation of acylfulvene compared to accumulation of illudin S (Table 5). There was also a markedly lower Vd coefficient for MGI 114.

Table 5 Analysis of cellular uptake using a nonlinear regression algorithm and the Kinetics software [29] (Vmax in picomoles/min for 10 million cells, Km in micromoles, Vd in min^{-1} for 10 million cells when substrate (S) is expressed in micromoles)

		MGI 114	Acylfulvene	Illudin S
HL60	Vmax	14	3.6	29.0
	Km	11	7.8	7.1
	Vd	<0.005	0.018	0.34
MV522	Vmax	18	333	35
	Km	25	16	6
	Vd	<0.005	0.013	0.13

Conversion of illudins to acylfulvene analogs results in a marked decrease in the in vivo toxicity as evidenced by the 50-fold higher MTD of acylfulvene and the 30-fold higher MTD of MGI 114 as compared to that of illudin S [10–14]. This large increase in MTD allows administration of tolerable therapeutic dosages of the acylfulvenes and leads to higher plasma concentrations [27, 28]. This in turn, based on cellular uptake parameters (Vmax, Km, and Vd), promotes increased uptake of acylfulvenes into tumor cells [30, 32]. The basis for this marked decrease in the in vivo toxicity of acylfulvenes is not fully understood. It may be related to the lower reactivity of acylfulvene to thiols and to enzymatic reduction with NADPH [34].

The presence of the methylhydroxyl group in MGI 114 results in a marked increase in the in vitro cytotoxicity as evidenced by a decrease in the IC_{50} values (2-h or 48-h exposure) in different human tumor cell lines, and by a decrease in the number of intracellular molecules per cell required to produce a 50% inhibition of cell survival (as determined by CFA) relative to acylfulvene. Despite a large variation between the in vitro toxicity of MGI 114, acylfulvene, and illudin S, the quantity of drug associated with cellular genomic DNA at equitoxic concentrations was relatively consistent for each cell line (Fig. 3). This suggests that the DNA damage produced by each agent was equivalent with respect to toxicity and repair. Why the addition of a single methylhydroxyl group increases the in vitro toxicity of acylfulvenes, without significantly altering in vivo toxicity, remains unknown. This methylhydroxyl group in MGI 114 may affect cellular toxicity by increasing the solubility of MGI 114 as compared to that of acylfulvene. Also, the primary allylic hydroxyl is quite reactive and may react with intracellular nucleophiles [34].

The results of this study does not exclude the possibility that additional mechanisms may contribute to the increased efficacy of MGI 114 such as variations in the rate of cellular repair of DNA damage, in vivo pharmacokinetic differences, in vivo distribution into normal

tissue, cellular metabolism that results in production of therapeutically active metabolites with different toxicities, or increased solubility. Further study is needed to determine if these mechanisms contribute in part to the increased efficacy of MGI 114.

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