Application of the MicroAmes Screen and MultiCASE SAR for Assessing the Genotoxic and Carcinogenic Potentials of the Pharmaceutical Impurity, Difluoroaniline

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Abstract

In the pharmaceutical industry, impurities from raw materials, intermediates, degradation products, synthetic reactants, and residual solvents should be assessed for genotoxic potential. Early in drug development, isolated impurities are often of insufficient quantity to fully test for genotoxicity and thus predictive screening tools become important for risk assessment. Difluoroaniline (DFA), a model impurity, was evaluated for genotoxicity by screening in the microAmes, an assay predictive for the full plate Ames test but requiring only 10 mg when used with 5 tester strains with or without an S9 metabolic activation system. DFA was also assessed for carcinogenicity using MultiCASE computer-assisted SAR coupled with ICSAS expert system. The National Toxicology Program in 1984 reported DFA to be negative in the Ames test with rat liver S9 but positive using 30% hamster S9. To understand the human relevance of this finding, DFA was evaluated in the microAmes screen using up to 30% human liver S9. The microAmes screen demonstrated DFA to be positive with hamster S9 at concentrations down to 10% and negative with rat S9 at concentrations up to 30%. DFA results using human S9 at concentrations up to 30% were also negative. These results indicate that activation of DFA by human liver S9 is more similar to rat and unlike the hamster. These results are supported by literature data indicating that hamster induced liver microsomes have higher rates of arylamine N-hydroxylation (activation step) and lower rates of arylamine ring hydroxylation (detoxication step) than rat microsomes. Using MultiCASE, the overall carcinogenicity potential of DFA via ICSAS expert system was also negative. These assessments demonstrate the utility of 1) using the microAmes screen for exploring the potential genotoxicity properties of impurities, particularly when the amount of an isolated impurity is limited; and 2) employing well validated SAR databases to evaluate impurities for their carcinogenic potential.

Introduction

2,4-Difluoroaniline (DFA) has been previously reported to be positive in the Ames test in the presence of a hamster S9 activation system but negative in the presence of rat S9 activation (Zeiger et al. 1988). Literature information indicates hamster liver fraction to have higher rates of arylamine N-hydroxylation (activation step) and lower rates of ring-hydroxylation of aniline (a detoxification pathway) relative to other species evaluated (Lotlikar and Hong 1981; Ikeda et al. 1972).

Methods – MultiCASE SAR

MultiCASE uses CASE (Computer Automated Structure Evaluation) and its successor, MCASE, to organize data and predict various toxicological endpoints on the basis of structural fragments. Mutagenicity potential of DFA was tested using A2H database representing Ames Salmonella mutagenicity data which includes FDA proprietary information. Carcinogenicity potential was tested using databases which contain FDA proprietary information for male rat (AG1), female rat (AG2), male mouse (AG3), and female mouse (AG4) with an overall carcinogenicity evaluation obtained through an experimental approach called ICSAS (Informatics and Computational Safety Analysis Staff) Decision Support Method. ICSAS is an applied regulatory research group within FDA-CDER: however, the answers from ICSAS do not constitute FDA guidance.

Methods - microAmes

The microAmes screen uses an agar format in multi-well plates and is a modification of the standard Salmonella/E. coli mammalian microsome assay (Ames et al. 1975/1981, Brusick et al. 1980, Maron and Ames 1983, Watanabe et al. 1998). As per the "full-plate" regulatory Ames assay, this screen uses an agar format, evaluates precipitation and cytotoxicity, and uses an equivalent top concentration (ICH and OECD guidelines). Because this is a "micronized" Ames assay, compound requirements are low (approx 10 mg) and turnaround time reduced (report within 2 weeks of receiving test article).

A preliminary assay was conducted to determine the optimal concentration of liver S9 fractions to use for activating 2,4-difluoroaniline (DFA, Sigma lot# 12110MD) in the microAmes screen. This evaluation used strains TA98 and TA100 treated with DFA and positive and negative controls. Positive control compounds were used at 4 different concentrations to ensure the assay system was sensitive to known mutagenic compounds. DMSO was used as the vehicle control. Activation systems were 1) Aroclor™ 1254 induced rat liver S9 2) Aroclor™ 1254 induced hamster liver S9, and 3) pooled human liver S9. S9 mixtures were at concentrations of 10, 20 or 30%.

Based on the results of this preliminary assay, a concentration of 20% was selected for the DFA microAmes screen. DFA was tested in duplicate with strains TA97a, TA98, TA100, TA1535 and WP2uvrApKM101 in the presence and absence of three different metabolic activation systems at 20% concentrations of the S9 activation systems using DFA concentrations of 0.25, 2.5, 12.5, 25, 75 and 250 µg/well.

Results

MultiCase SAR program: DFA was predicted not to be mutagenic in Ames Salmonella strains. Overall carcinogenicity potential via ICSAS expert system was negative as were each of the 4 carcinogenicity databases.

MicroAmes Positive and Vehicle Controls: DFA and positive control (2-aminoathracene) responses were most sensitive without cytotoxicity at 20% S9 (Figure 1 displays results for positive control). Vehicle and positive controls for all strains yielded the expected results demonstrating that the microAmes screen was sufficiently sensitive to detect mutagens. The S9 fractions from all three species were sensitive in activating the positive control mutagen 2 aminoanthracene; however, human liver S9 appeared to be more sensitive than either hamster or rat liver S9 (see Figure 2 for TA98 and TA100).

MicroAmes 2,4-Difluoroaniline Results: Results are summarized in Figure 3. No noteworthy cytotoxicity (reduction in background lawn and/or number of revertant colonies) or precipitates were observed with any of the conditions. An increase in the revertant colonies was observed at >75 µg/well in strains TA98 and TA100 with hamster liver S9. No meaningful increases in revertant colonies were observed in any other strain with any of the conditions.

Overall Results

Parameter	Results
MutiCase SAR	
Ames Mutagenicity	-
Carcinogenicity	-
MicroAmes Screen	
Rat	-
Hamster	+
Human	-

FIGURE 1
TA100: Positive Control (2-Aminoanthracene)
Responses with 10%, 20%, and 30% Human S9 Activation System

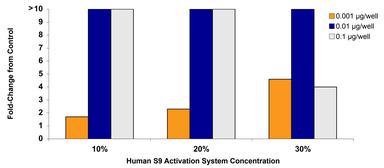


FIGURE 2A
TA98: Positive Control (2-Aminoanthracene)
Responses with Rat, Hamster and Human S9 Activation System (20%)

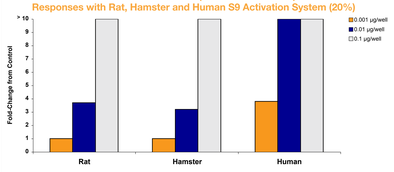


FIGURE 2B
TA100: Positive Control (2-Aminoanthracene)
Responses with Rat, Hamster and Human S9 Activation System (20%)

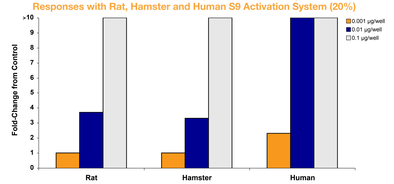
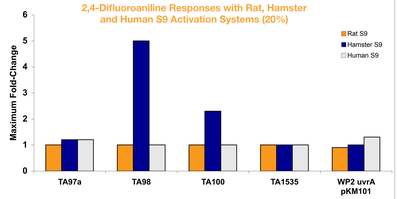


FIGURE 3
2,4-Difluoroaniline Responses with Rat, Hamster and Human S9 Activation Systems (20%)



Conclusions

2,4-Difluoroaniline (DFA) was negative for mutagenicity and carcinogenicity when structure was evaluated using MultiCASE A2H Ames Salmonella Mutagenicity database and using the ICSAS expert system coupled with AG1, AG2, AG3 and AG4 rodent carcinogenicity databases. DFA induced a positive response in strains TA98 and TA100 using a hamster liver S9 mix in this microAmes screen. Results using rat and human liver S9 activation systems yielded negative results for all five tester strains.

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