Ku and ATM appear to be required for synergy between tirapazamine and cisplatin

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Abbreviations: TPZ, Tirapazamine; CP, Cisplatin; MMR, Mismatch repair; C_I, Combination Index; F_a, Fraction affected

Abstract

Tirapazamine (TPZ) generates single and double strand breaks under hypoxic conditions. TPZ potentiates the cytotoxicity of cisplatin (CP) in a synergistic manner and is currently being evaluated in combination with CP and other chemotherapeutic agents in Phase III clinical trials. We have evaluated the interaction between TPZ and CP in a variety of cell lines using the Chou and Talalay median effect analysis. We found that TPZ and CP were antagonistic in the CP-sensitive A2780 human ovarian carcinoma cell line, but were highly synergistic in the CP-resistant A2780/CP cell line. We compared the interaction between TPZ and CP in the MMR-deficient HCT116 human colon carcinoma cell line and its MMR-proficient, chromosome 3 complement HCT116 + chr3. In both cell lines, the TPZ-CP combination was antagonistic at low drug concentrations and synergistic at high drug concentrations. Thus, the synergistic interaction between TPZ and CP in the A2780/CP cell line does not appear to result from the MMR deficiency. Because TPZ treatment must precede CP treatment for synergy, it appeared likely that strand break formation is necessary for synergy. Thus, we also compared the interaction between TPZ and CP in CHO cells with Ku (AA8) and without Ku (Xrs6). The TPZ-CP combination was synergistic in the Ku-proficient AA8 cells and antagonistic in the Ku-deficient Xrs6 cells. Finally, we compared the interaction between TPZ and CP in a normal human fibroblast cell line (GM01815) and the ATM-deficient cell line (GM09582). The TPZ-CP combination was synergistic in GM01815 cells and antagonistic in GM09582 cells. These data suggest that the Ku-ATM signaling pathway may be required for synergy between TPZ and CP.

Keywords: Tirapazamine, Cisplatin, Synergy, ATM, Ku, Mismatch repair

1. Introduction

Tirapazamine (3-amino-1,2,4-benzotriazine 1,4-di-*N*-oxide; SR 4233) (TPZ) is a chemotherapeutic agent that is converted to a cytotoxic species at low oxygen levels [1,2]. The cytotoxicity of TPZ results from activation by reductive enzymes in the nucleus that involves a one-electron reduction of the drug to a radical that kills hypoxic cells by hydrogen abstraction from DNA [2]. The much lower toxicity in aerobic cells results from oxidation of the radical back to the nontoxic parent compound. Under hypoxic conditions TPZ produces base damage, DNA single strand breaks, double strand breaks, and chromosome aberrations [2-5]. Cisplatin (*cis*-diaminedichloroplatinum(II)) (CP) has been broadly used for cancer treatment since 1978 and exerts its cytotoxic effects through binding to DNA [6]. CP forms intra- and interstrand DNA adducts at GG, AG, and GNG sequences [7]. Clinical resistance to CP is multifactorial [8-11], with increased cellular proficiency of DNA intra- and interstrand crosslink repair, decreased drug accumulation, increased replicative bypass, and defects in the signaling pathways leading to apoptosis all affecting CP resistance. TPZ is currently in Phase III clinical trials in combination with CP and radiation [12,13]. These trials have shown that TPZ can produce a therapeutic gain by potentiating the efficacy of CP without changing its systemic toxicity [14-16]. However, it has also been shown that both agents produce a mild leukopenia, and when they are administered in combination, this effect is additive [16].

Synergistic interactions between TPZ and CP depend upon the time interval between drug incubations. The interaction is no more than additive when the two drugs are given

simultaneously or if CP is given prior to TPZ, but an extra 4-5 logs of cell kill can be produced when TPZ incubation precedes CP incubation by at least 1 hour, suggesting that TPZ sensitizes the cells to CP cell killing [16]. The synergy between TPZ and CP is optimal when the TPZ treatment occurs under hypoxic conditions ($[O_2] < 1\%$) [17]. However, it has been shown that significant potentiation of CP cytotoxicity occurs even at oxygen concentrations at which TPZ alone is not cytotoxic, suggesting that even very low levels of TPZ-induced damage are sufficient to potentiate CP cytotoxicity in tumor cells [17]. Since TPZ does not effect the systemic toxicity of CP, with the exception of mild leukopenia, essentially all of the additional cell kill translates into a therapeutic gain for this combination [18].

The mechanism(s) for synergistic interactions between TPZ and CP are not presently known. Kovacs *et al.* [17] have proposed that some of the potentiation of CP cell kill by TPZ is caused by an increased incidence of CP interstrand crosslinks due to inhibition of repair. Similarly, Goldberg *et al.* [19] found that cells repair CP interstrand crosslinks in the 48 hours following exposure, but cells that were pretreated with TPZ showed substantially reduced repair capacity. This hypothesis is strengthened by data demonstrating that cell lines lacking XPF or ERCC1 show substantially less synergy than parental cell lines [19]. However, the mechanism of this effect is unclear. Synergy could result from difficulty in the repair of compound lesions (e.g. adjacent strand breaks and crosslinks), the competition between strand break repair and interstrand crosslink repair for the same proteins, or a strand break-induced damage response that recruits essential repair proteins from interstrand crosslink repair to strand break repair. It is also not clear

whether the effects of TPZ on interstrand crosslink repair are sufficient to explain all of the synergy between TPZ and CP. Thus, it is important to evaluate other potential mechanisms of synergy. For example, the synergistic interaction between TPZ and CP could also result in part from the fact that TPZ and CP cause apoptosis through parallel, but distinct signaling pathways. Double strand breaks induce apoptosis via signaling pathways involving both Ku, ATM, and p53 and Ku, ATM, cAbl, and p73 [20,21]. CP, on the other hand, appears to cause apoptosis via a signaling pathway involving ATR and p53 [22,23]. Furthermore, the sites of phosphorylation of p53 by ATM and ATR are different [24]. Thus, it is possible that cross talk between these pathways produces a synergistic cytotoxicity. Finally, it has also been proposed that CP sensitizes tumor cells to ionizing radiation via the inhibition of DNA-PK phosphorylation of target proteins resulting in a defect in double strand break repair [25,26]. This inhibition of repair could also contribute to the synergy seen with TPZ and CP, but this would appear less likely because synergy is greatest when TPZ treatment precedes CP treatment.

In order to elucidate the mechanism(s) of synergy between TPZ and CP, we measured cell survival following TPZ and CP treatment in a variety of cell lines that were proficient and deficient in several different repair and/or signaling proteins. The first pair of cell lines that we examined was a human ovarian carcinoma A2780 cell line and its CP-resistant counterpart, A2780/CP. We found that the CP-TPZ combination was antagonistic in the CP-sensitive A2780 cells but was strongly synergistic in the CP-resistant A2780/CP cells. Resistance of the A2780/CP cell line may be explained by several mechanisms including a deficiency in mismatch repair (MMR) [27,28]. In order

to elucidate if the MMR pathway contributed to synergy, we examined the interaction between TPZ and CP in MMR-deficient and MMR-proficient cell lines. The A2780/CP cell line has also been shown to have mutant p53 that is selectively defective in its response to CP. In response to CP treatment p53 accumulation, G1 arrest, and apoptosis are all significantly decreased in the A2780/CP cell line, yet all three parameters respond normally to ionizing radiation and other types of DNA damage [22]. Since TPZ is equally cytotoxic in p53(+/+) and p53(-/-) cells [29], we also determined whether the signaling pathway leading from TPZ-induced double strand breaks to apoptosis might contribute to the synergy between TPZ and CP.

2. Materials and Methods

2.1 Materials and Reagents. TPZ was obtained from Sanofi-Synthelabo Research Division (Great Valley, PA), and all other reagents were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated. Hypoxic conditions were achieved in a Billups-Rothenberg model MIC-101 modular incubation chamber (Billups-Rothenberg, Del Mar, CA). Oxygen concentration was measured with a YSI model 55CE dissolved oxygen probe (YSI, Marion, MA). The hypoxia chamber with oxygen probe was mounted on a motorized tilting apparatus.

2.2 Cell lines and Cell culture. The CP-sensitive A2780 and CP-resistant A2780/CP cell lines were generously provided by Dr. Paul Andrews (Georgetown University, Washington, DC). The MMR-deficient HCT116 and MMR-proficient HCT116 + chr3 cell lines were generously provided by Dr. Tom Kunkel (National Institute of

Environmental Health and Sciences, Research Triangle Park, NC). Ku-proficient AA8 and Ku-deficient Xrs6 cell lines were generously provided by Dr. Dale Ramsden (University of North Carolina at Chapel Hill, Chapel Hill, NC). ATM-proficient GM01815 and the ATM-deficient GM09852 cell lines were generously provided by Dr. William Kaufmann (University of North Carolina at Chapel Hill, Chapel Hill, NC). All cell lines were maintained in a humidified environment with 95% air and 5% CO_2 at 37°C and in media supplemented with 10% FCS and penicillin-streptomycin (5 units/L penicillin G sodium and 5 μg/L streptomycin sulfate, Gibco). A2780 and A2780/CP cells were maintained in RPMI 1640. HCT116 + chr3 cells were maintained in DMEM + 400 μg/mL G418. HCT116 cells, GM01815 cells, and GM09852 cells were maintained in DMEM. AA8 cells were maintained in α-MEM. Xrs6 cells were maintained in F12 (HAM).

2.3 Cytotoxicity Determination. Exponentially growing cells were plated in 35 mm glass culture dishes at 1 x 10^6 cells/dish. After an overnight incubation to allow for attachment, the cells were washed with PBS, and 1 mL of media without FCS was added. Various amounts of TPZ were added and the cells were incubated for 1.5 hours at 37°C under hypoxic conditions (5% CO_2 and 95% N_2; O_2 concentration < 0.1 mg/L) in the Billups-Rothenberg modular incubation chamber. Throughout the incubation the motorized tilting apparatus was rotating so that the cells would be continually covered by media. Following incubation the cells were washed with PBS, trypsinized, and plated in 4 mL media at cell densities varying from 100 cells/well to 100,000 cells/well in 6 well plates,

depending on the drug concentration used. After 7-10 days, cells were fixed to the plates with a 3:1 methanol:acetic acid solution and then stained with 0.04% Giemsa stain (Fisher). Colonies consisting of >50 cells were counted and the IC_{50} was determined from the semilog plot of cell number versus drug concentration. The IC_{50} for CP was determined in the same manner except that cells were treated with CP in 4 mL of media containing 10% FCS, the drug incubation period was 1 hour, and aerobic conditions (5% CO_2 and 95% O_2) were used during treatment. The GM01815 and GM09852 cells were centrifuged for 10 min at 5000 rpm and the pellet was rinsed with PBS. The cell pellet was resuspended in 4 mL of media and plated as described above. Dead cells were excluded from the count with the use of Trypan Blue (Fisher).

2.4 Synergy Determination. For synergy determinations cells were incubated with varying concentrations of TPZ under hypoxic conditions in 1 mL of media without FCS for 1.5 hours, washed with PBS, and incubated in 4 mL of fresh media containing 10% FCS for 1 hour under aerobic conditions. The cells were then washed with PBS and incubated with varying concentrations of CP for 1 hour with 4 mL of media containing 10% FCS. Following incubation the cells were washed with PBS, trypsinized, and replated in 4 mL media in 6 well plates. The relative concentrations of TPZ and CP were maintained at the molar ratio of their IC_{50}'s as recommended by Chou and Talalay [30]. Following drug treatment cell survival was measured by the colony formation assay as described above. The interaction between TPZ and CP was analyzed by the Chou and Talalay median effect principle [30] using the Calcusyn program (Biosoft, Ferguson MO). The program calculates a combination index (C_I) that is used to identify

synergistic, additive, and antagonistic drug interactions. The type of interaction is determined by plotting C_I versus the fraction of cells affected (F_a) by increasing drug concentrations, where the drugs are added at the molar ratio of their IC_{50}.

3. Results

3.1 Cytotoxicity Determination. The results of the cytotoxicity determination experiments are summarized in Table I. As expected the CP resistant cell line A2780/CP showed a 1.8 fold resistance to CP relative to the parental A2780 cell line, but was twice as sensitive to TPZ as the CP sensitive A2780 cell line. In the MMR-deficient cell line HCT116, a minor resistance to CP (1.2-fold) was seen, and it was only slightly more sensitive to TPZ than its MMR-proficient, chromosome 3 complement HCT116 + chr3. The Ku-deficient Xrs6 cell line was 8.4-fold more sensitive to TPZ than the Ku-proficient AA8 cell line which confirmed that our conditions of incubation were sufficiently hypoxic for TPZ to generate double strand breaks. Interestingly, the Xrs6 cell line was also 9.6-fold more sensitive to CP than the AA8 cell line. The reason for this effect is not known, but it has been proposed that double strand breaks could form as a result of aberrant repair of platinum interstrand crosslinks [31]. The ATM-deficient GM09852 cell line was 4.6-fold more sensitive to CP than the ATM-proficient GM01815 cell line as reported previously [24], but it was 1.8-fold more resistant to TPZ.

3.2 Interactions between TPZ and CP in CP-sensitive and CP-resistant human ovarian carcinoma cell lines. The interactions between TPZ and CP were determined by the

median effect analysis developed by Chou and Talalay [30]. Fraction affected (F_a) is defined as the fraction of cells affected by a given dose of a drug or drug combination. The combination index (C_I) equation is based on the multiple drug-effect equation of Chou-Talalay derived enzyme kinetic models, where C_I <, =, and > 1 indicates synergism, additive effect, and antagonism, respectively [30]. The effective dose (ED) is defined as the combined drug dose at which a defined percentage of cells are killed (i.e. 90% of the cells are killed at ED_{90}). The relative concentrations of TPZ and CP were maintained at the molar ratio of their IC_{50}'s because this is the experimental design recommended by Chou and Talalay [30]. The plots of C_I versus F_a for the A2780 and A2780/CP cell lines are shown in Figure 1, and the C_I at the ED_{50}, ED_{75}, and ED_{90} combined drug doses are shown in Table II. These data clearly indicate that the CP-TPZ combination is antagonistic in the CP-sensitive A2780 cells, but is strongly synergistic in the CP-resistant A2780/CP cells. The synergy between TPZ and CP in the A2780/CP cell line has been reported previously [32], but the interaction between TPZ and CP in the parental A2780 cell line had not previously been investigated. The synergy seen in the A2780/CP cell line is unusual in that it is seen over the entire range of drug conditions tested.

3.3 Interactions between TPZ and CP in MMR-proficient and MMR-deficient human colon carcinoma cell lines. Since the A2780/CP cell line is MMR-deficient [27,33], we compared the interaction between TPZ and CP in the MMR-deficient HCT116 human colon carcinoma cell line and its MMR-proficient, chromosome 3 complement HCT116 + chr3. The plots of C_I versus F_a are shown in Figure 2, and the C_I at the ED_{50}, ED_{75}, and

ED_{90} combined drug doses are shown in Table II. In both the MMR-proficient HCT116 + chr 3 cell line and the MMR-deficient HCT116 cell line the CP-TPZ combination is antagonistic at low drug concentrations and synergistic at high drug concentrations.

3.4 Interactions between TPZ and CP in Ku-proficient and Ku-deficient Chinese hamster ovary cells. The A2780/CP cell line has mutant p53, which decreases the apoptotic response to CP treatment [22]. However, the cell line retains a normal apoptotic response to ionizing radiation and other DNA-damaging agents [22]. Thus, to investigate whether the double strand break-induced signaling pathway was involved in apoptosis, we first compared the interaction between TPZ and CP in the Ku-deficient Xrs6 cell line and the Ku-proficient AA8 cell line. The plots of C_I versus F_a are shown in Figure 3, and the C_I at the ED_{50}, ED_{75}, and ED_{90} combined drug doses are shown in Table II. In the AA8 cell line the CP-TPZ combination is slightly synergistic at low drug concentrations (C_I=0.92 at F_a=0.23), becomes antagonistic at intermediate drug concentrations, and then becomes strongly synergistic at high drug concentrations (C_I=0.55 at F_a=0.995 and 0.999). Thus, according to the criteria established by Chou and Talalay, the CP-TPZ combination is considered to have overall synergy in the AA8 cell line. However, the synergy appears to be dependent on high levels of DNA damage. In contrast, the CP-TPZ combination is clearly antagonistic at all drug concentrations in the Ku-deficient Xrs6 cell line.

3.5 Interactions between TPZ and CP in ATM-proficient and ATM-deficient human fibroblast cell lines. The effect of Ku on the synergy between TPZ and CP could indicate that either repair of the double strand breaks themselves was required for synergy or that

a double strand break-induced signaling pathway was required for synergy. Thus, we also compared the interaction between TPZ and CP in the ATM-deficient GM09852 cell line and the ATM-proficient GM01815 cell line. The plots of C_I versus F_a are shown in Figure 4, and the C_I at the ED_{50}, ED_{75}, and ED_{90} combined drug doses are shown in Table II. These data clearly show that the CP-TPZ combination is antagonistic in the ATM-deficient GM09852 cells but is strongly synergistic in the ATM-proficient GM01815 cells. The synergy seen in the GM01815 cell line is similar to that seen in the A2780/CP cell line, in that it was maintained at all drug concentrations.

4. Discussion

4.1 Interactions between TPZ and CP in CP-sensitive and CP-resistant human ovarian carcinoma cell lines. The reason for the strong synergy seen in the A2780/CP cell line is not clear at present. However, because the synergy is not seen in the parental A2780 cell line, it appears likely that the synergy depends on one or more of the resistance mechanisms of the A2780/CP cell line. CP resistance in the A2780/CP cell line is associated with decreased accumulation and increased efflux of CP [34], increased glutathione levels [34], increased repair of both intra- and interstrand crosslinks [34,35], increased post-replication repair (replicative bypass) [36], decreased MMR [27,33], and a mutant p53 that interferes with CP-induced G1 arrest and apoptosis [22]. Certainly, the ability of TPZ to interfere with the repair of CP interstrand crosslinks [19, 20] could contribute to some of the synergy between TPZ and CP in that cell line. However, it is unlikely to be responsible for all of the synergy because increased repair appears to make a relatively small contribution to the overall resistance of the A2780/CP cell line [34,35].

The A2780/CP cell line is more sensitive to TPZ than the A2780 cell line, so it is also possible that the A2780/CP cells have lost some of their capacity for double strand break repair.

The A2780/CP cell line is also defective in MMR. The MMR complex binds to CP intrastrand GG adducts [37,38], but is not normally involved in the repair of these adducts because of the absence of nearby strand breaks to initiate the repair process. If TPZ were to induce single strand breaks in the vicinity of CP adducts, the MMR system might become involved in the removal of CP adducts from the DNA. If that were the case, defects in MMR could also contribute to the synergy observed between TPZ and CP in the A2780/CP cell line.

Finally, the A2780/CP cell line has a defect in p53 that interferes with p53 accumulation, G1 arrest, and apoptosis in response to CP treatment, but not in response to ionizing radiation and other types of DNA damage [22]. Because TPZ causes double strand breaks it is likely to induce apoptosis by a parallel, but distinct signaling pathway than the one that is activated following CP damage [20-23]. In fact, the induction of apoptosis by TPZ is independent of p53 status [29]. Thus, cross talk between the TPZ-induced and CP-induced signaling pathways leading to apoptosis could be responsible for some of the synergy between these drugs, especially in cell lines such as A2780/CP that lack the p53-dependent apoptotic pathway in response to CP damage. Whatever the mechanism of synergy in the A2780/CP cell line, if the same level of synergy is seen in other CP resistant cell lines it would be of potential therapeutic interest.

4.2 Interactions between TPZ and CP in MMR-proficient and MMR-deficient human colon carcinoma cell lines. As described above, if TPZ and CP were to form compound lesions containing both strand breaks and intrastrand crosslinks it is theoretically possible that MMR could contribute to the repair of those compound lesions. If this were the case, defects in MMR would decrease the repair of both lesions and would, therefore, increase the synergy between TPZ and CP. In both the MMR-proficient HCT116 + chr 3 cell line and the MMR-deficient HCT116 cell line the CP-TPZ combination was antagonistic at low drug concentrations and synergistic at high drug concentrations. The degree of synergy seen at high drug concentrations with the MMR-deficient cell line was slightly greater than that seen with the MMR-proficient cell line (Table II), but was clearly not sufficient to explain the high degree of synergy observed at all drug concentrations in the A2780/CP cell line. Therefore, our results show that MMR does not make a significant contribution to synergy between TPZ and CP.

4.3 Interactions between TPZ and CP in Ku-proficient and Ku-deficient Chinese hamster ovary cells. As described above, it is also theoretically possible that the signaling pathway leading from double strand breaks to apoptosis may contribute to the synergy between TPZ and CP. Ku binds to double strand breaks and recruits both XRCC4, ligase IV, and ATM to the complex [39]. The role of ATM in double strand break repair itself is uncertain, but it has been postulated that ATM may serve to monitor the progress of double strand break repair and activate damage-response pathways if the repair is delayed or unsuccessful [40]. Since Ku appears to be involved in the original recruitment of ATM

to the double-strand break, it was logical to investigate the effect of Ku on the synergy between TPZ and CP. Our data suggest that Ku makes a contribution to this synergy. These data are consistent with the hypothesis that double strand breaks and CP adducts trigger apoptosis via independent pathways [20-23] and that the overall effects of the signaling pathways is synergistic rather than additive. However, the data would also be consistent with our hypothesis that the repair of TPZ-induced double strand breaks alone contributed to the synergy, perhaps by competing with nucleotide excision repair for common repair proteins.

4.4 Interactions between TPZ and CP in ATM-proficient and ATM-deficient human fibroblast cell lines. ATM is a key element in signaling pathways leading from double strand breaks to cell cycle checkpoints, enhanced repair, and apoptosis [20,21]. Furthermore, ATM is known to be recruited to double strand breaks in a Ku-dependent manner [39]. Our data clearly show that ATM is required for synergy between TPZ and CP. Thus, in cells with both Ku and ATM, there is synergy between TPZ and CP, but in cells that lack either one of these proteins, the combination is antagonistic. These data strongly suggest that the Ku-ATM signaling pathway is required for synergy between TPZ and CP. The relationship between these data and previous data [17,19] that suggest a relationship between TPZ treatment and repair of CP interstrand crosslinks is unclear at this time. One could postulate that activation of the Ku-ATM signaling pathway and decreased interstrand crosslink repair make independent contributions to the synergy between TPZ and CP. Alternatively, the Ku-ATM signaling pathway may affect the rate of interstrand crosslink repair. Further studies will be required to distinguish between

these possibilities. In any case, these data suggest that the ATM status of the tumor may affect the efficacy of the TPZ and CP combination.

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Table I. IC_{50} values ± standard error of the mean for tirapazamine and cisplatin cytotoxicity in all cell lines used. The number of repeats are shown in parentheses.

Table II. Combination indices (C_I) for the tirapazamine-cisplatin interactions in all cell lines used. Values for the C_I are given at the ED_{50}, ED_{75}, and ED_{90}, or the drug dosage required to kill 50%, 75%, and 90% of cells, respectively.

Fig. 1. The Interaction Between TPZ and CP in CP-Sensitive and CP-Resistant Cell Lines. Fig. 1A. Combination index versus fraction affected for the treatment of the CP-sensitive cell line A2780 with TPZ and CP. A molar ratio of 3.75:1 (TPZ/CP) was used. The data presented are an average of three experiments +/- standard error of the mean. 1B. Combination index versus fraction affected for the treatment of the CP-resistant cell line A2780/CP with TPZ and CP. A molar ratio of 1:0.74 (TPZ/CP) was used. The data presented are an average of three experiments +/- standard error of the mean. The best fit lines were determined by Calcusyn (Biosoft, Ferguson MO). The relative concentrations of TPZ and CP were maintained at the molar ratio of their IC_{50}'s because this is the most efficient experimental design according to Chou and Talalay [30].

Fig. 2. The Effect of Mismatch Repair Status on the Interaction Between TPZ and CP. Fig. 2A. Combination index versus fraction affected for the treatment of the MMR-proficient cell line HCT116 + chr3 with TPZ and CP. A molar ratio of 1:1.33 (TPZ/CP) was used. The data presented are an average of four experiments +/- standard error of the mean. 2B. Combination index versus fraction affected for the treatment of the MMR-

deficient cell line HCT116 with TPZ and CP. A molar ratio of 1:3.66 (TPZ/CP) was used. The data presented are an average of four experiments +/- standard error of the mean. The best fit lines were determined by Calcusyn (Biosoft, Ferguson MO). The relative concentrations of TPZ and CP were maintained at the molar ratio of their IC_{50}'s because this is the most efficient experimental design according to Chou and Talalay [30].

Fig. 3. The Effect of Ku on the Interaction Between TPZ and CP. Fig. 3A. Combination index versus fraction affected for the treatment of the Ku-proficient cell line AA8 with TPZ and CP. A molar ratio of 1:1.88 (TPZ/CP) was used. The data presented are an average of three experiments +/- standard error of the mean. 3B. Combination index versus fraction affected for the treatment of the Ku-deficient cell line Xrs6 with TPZ and CP. A molar ratio of 1:1.56 (TPZ/CP) was used. The data presented are an average of four experiments +/- standard error of the mean. The best fit lines were determined by Calcusyn (Biosoft, Ferguson MO). The relative concentrations of TPZ and CP were maintained at the molar ratio of their IC_{50}'s because this is the most efficient experimental design according to Chou and Talalay [30].

Fig. 4. The Effect of ATM on the Interaction Between TPZ and CP. Fig. 4A. Combination index versus fraction affected for the treatment of the ATM-proficient cell line GM01815 with TPZ and CP. A molar ratio of 1:16 (TPZ/CP) was used. The data presented are an average of six experiments +/- standard error of the mean. 4B. Combination index versus fraction affected for the treatment of the ATM-deficient cell line GM09582 with TPZ and CP. A molar ratio of 1:1.73 (TPZ/CP) was used. The data

presented are an average of six experiments +/- standard error of the mean. The best fit lines were determined by Calcusyn (Biosoft, Ferguson MO). The relative concentrations of TPZ and CP were maintained at the molar ratio of their IC_{50}'s because this is the most efficient experimental design according to Chou and Talalay [30].

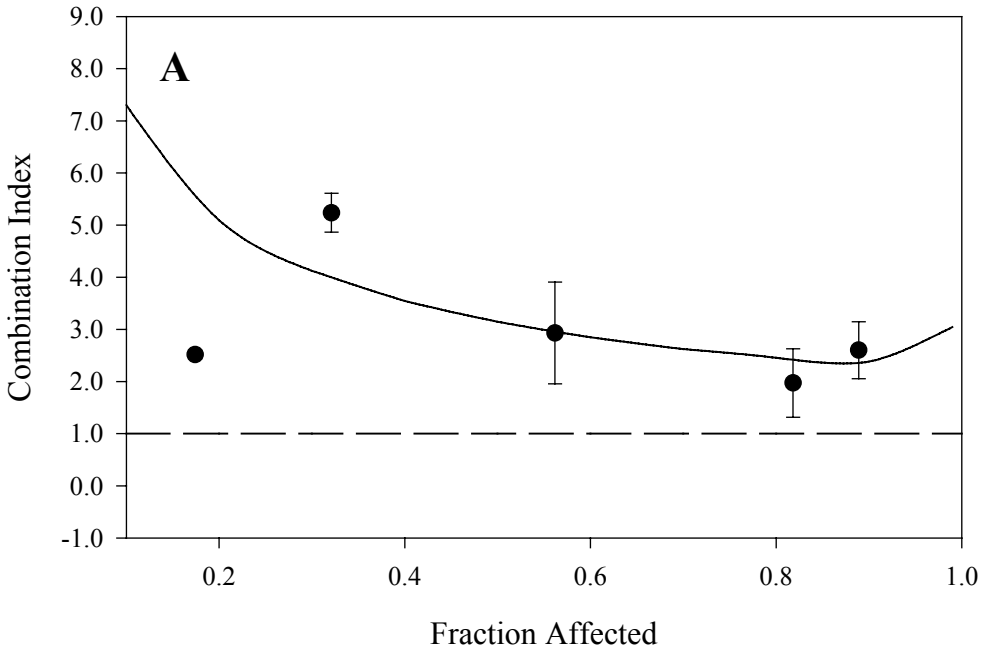
Table I. IC_{50} values ± standard error of the mean for tirapazamine and cisplatin cytotoxicity in all cell lines used. The number of repeats are shown in parentheses.

	IC_{50}	
Cell Line	**Tirapazamine**	**Cisplatin**
A2780	7.0 ± 1.6 μM (3)	1.9 ± 0.2 μM (3)
A2780/CP	3.6 ± 0.6 μM (3)	3.5 ± 0.2 μM (3)
HCT116 + chr3	5.8 ± 2.4 μM (4)	7.6 ± 1.1 μM (4)
HCT116	5.7 ± 1.7 μM (4)	9.2 ± 0.4 μM (4)
AA8	7.9 ± 1.2 μM (3)	13.4 ± 1.0 μM (3)
Xrs6	0.94 ± 0.09 μM (4)	1.4 ± 0.3 μM (4)
GM01815	2.2 ± 0.4 μM (6)	35.3 ± 2.1 μM (6)
GM09852	3.9 ± 0.6 μM (6)	7.7 ± 0.7 μM (6)

Table II. Combination indices for the tirapazamine-cisplatin interactions in all cell lines used.

	Combination Index (C_I) at:		
	ED_{50}	**ED_{75}**	**ED_{90}**
A2780	3.1	2.5	2.4
A2780/CP	.51	.49	.48
HCT116 + chr 3	1.3	0.94	0.74
HCT116	1.5	0.54	0.38
AA8	1.07	0.98	0.89
Xrs6	1.6	1.6	1.6
GM01815	0.44	0.19	0.08
GM09852	1.4	1.2	1.2

Figure 1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Fraction Affected
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7.0
6.0
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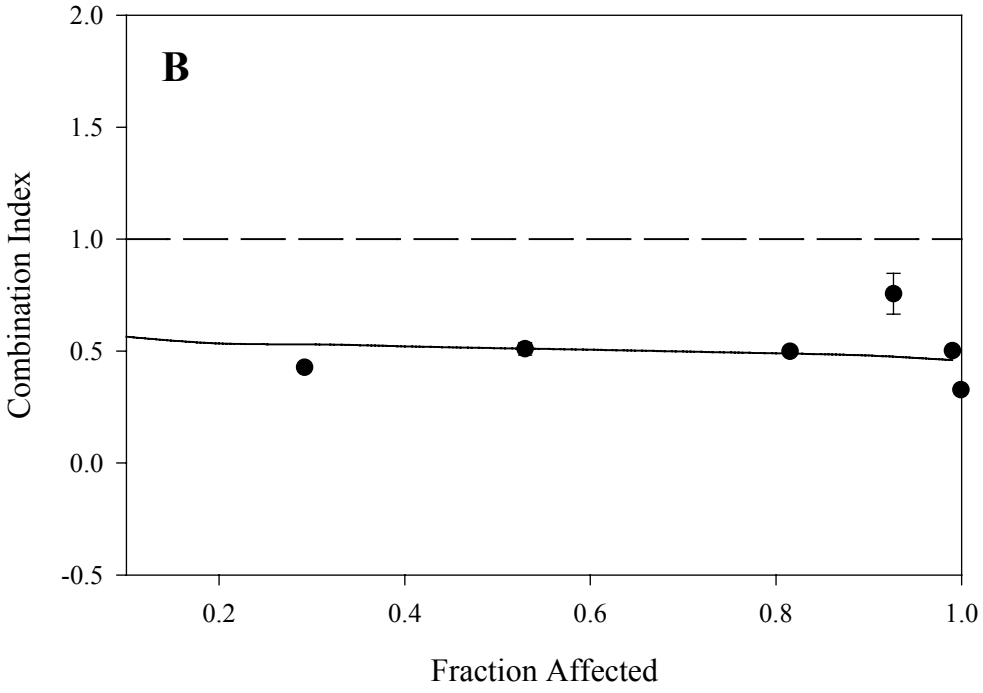
0.2
0.4
0.6
0.8
1.0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Combination Index
Fraction Affected
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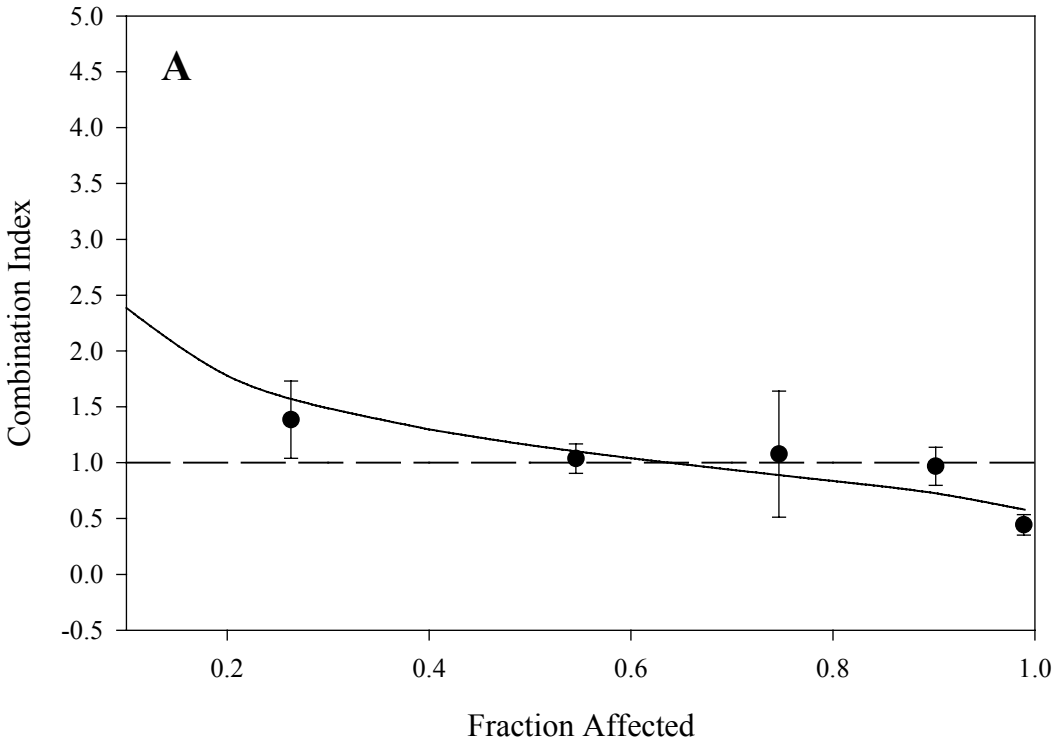
1.0

Figure 2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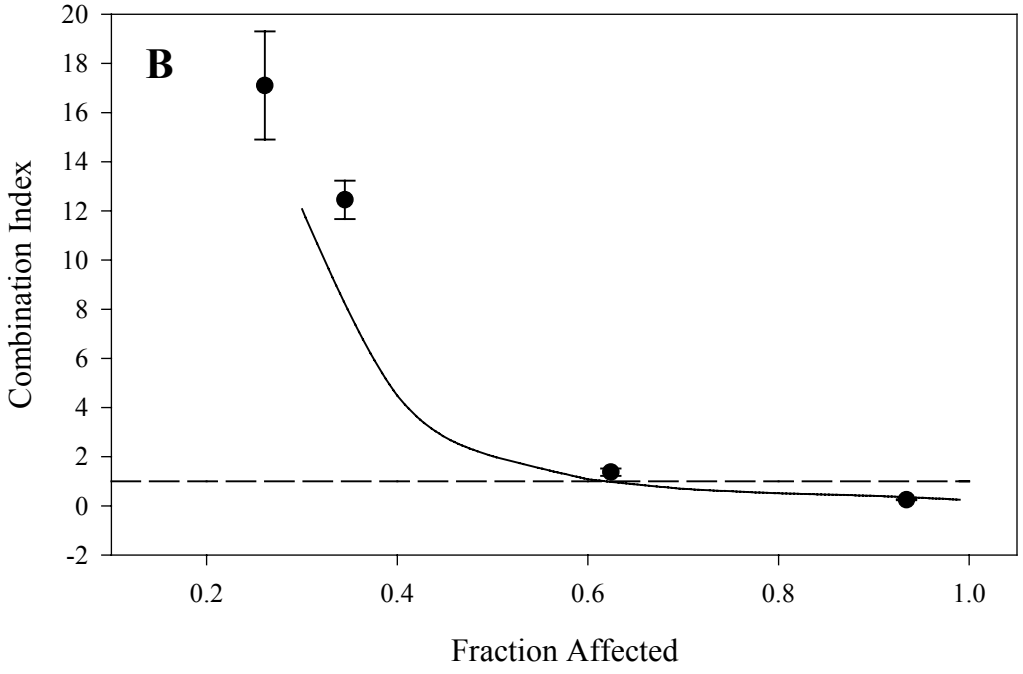
0.6
0.8
1.0


B
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Fraction Affected
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10
8
6
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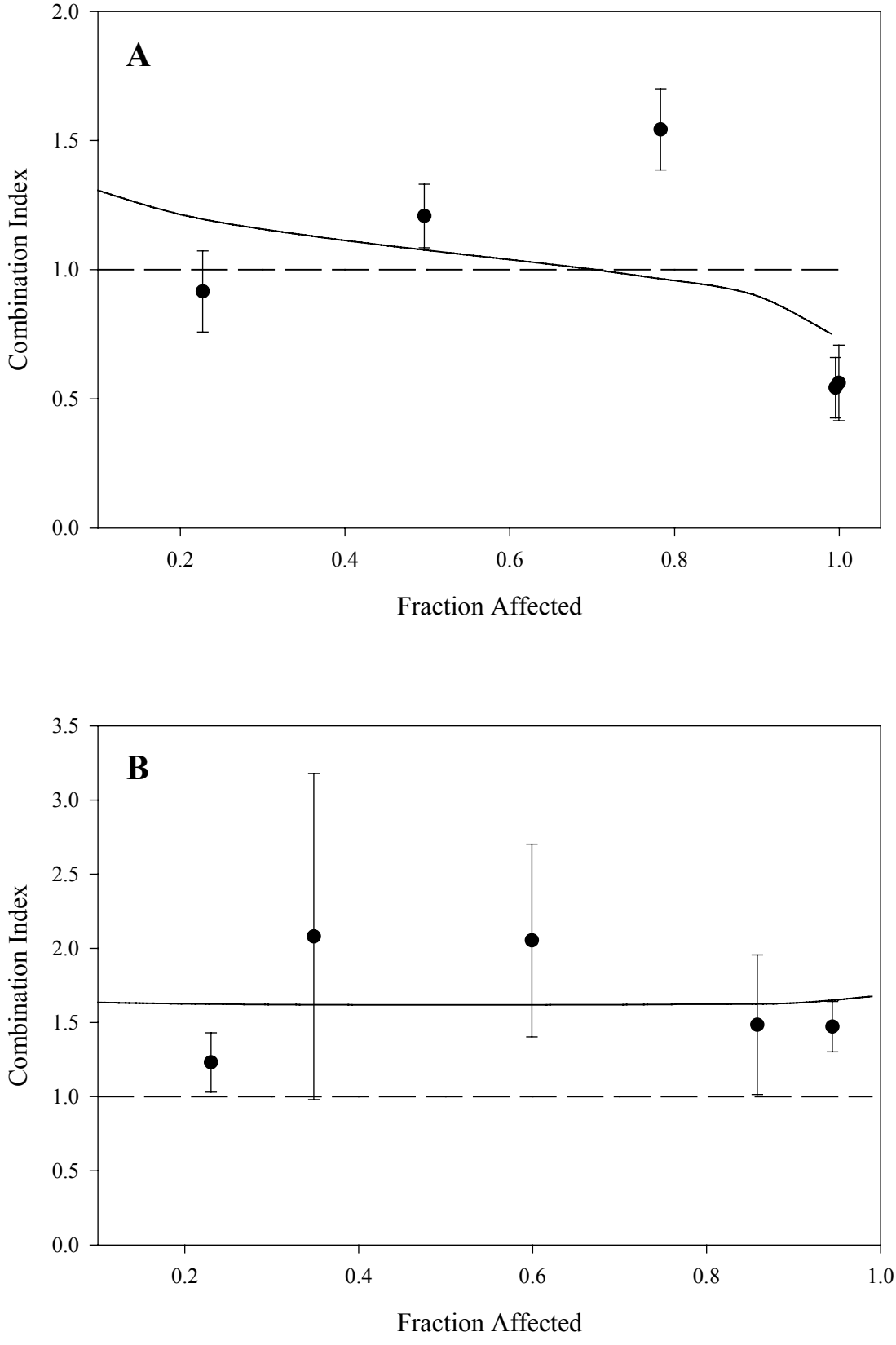
0.6
0.8
1.0

Figure 3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Fraction Affected
B
Combination Index
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3.0
2.5
2.0
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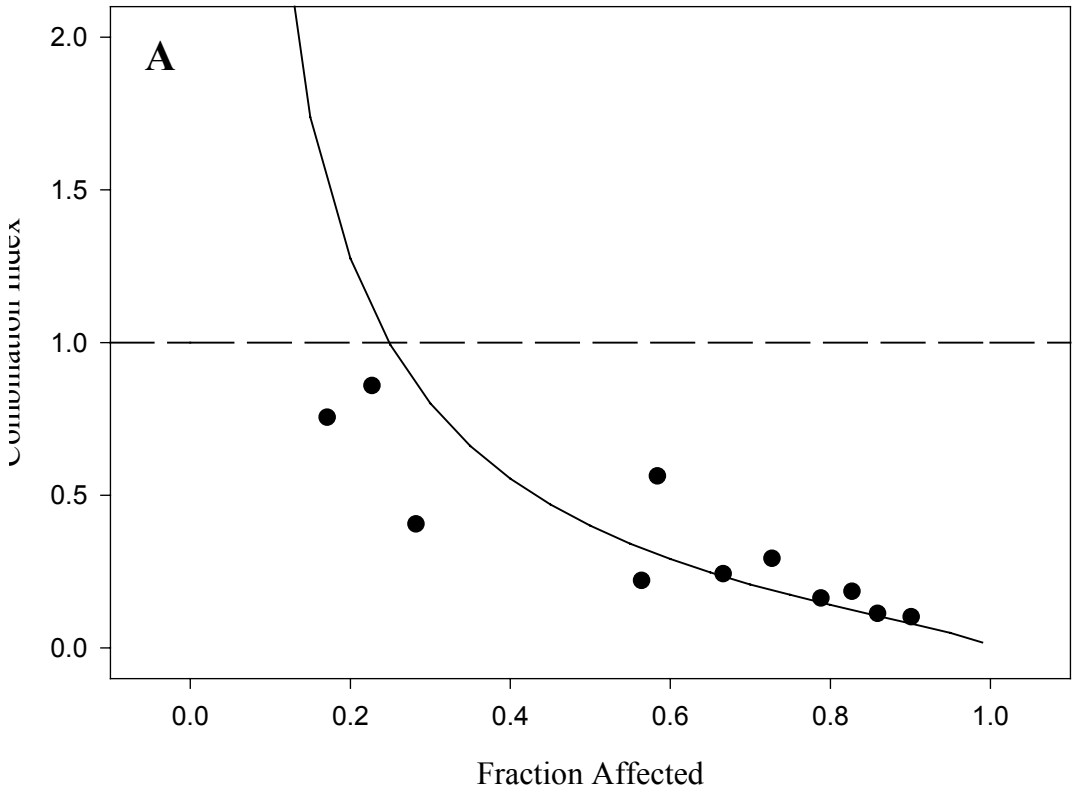
Fraction Affected

Figure 4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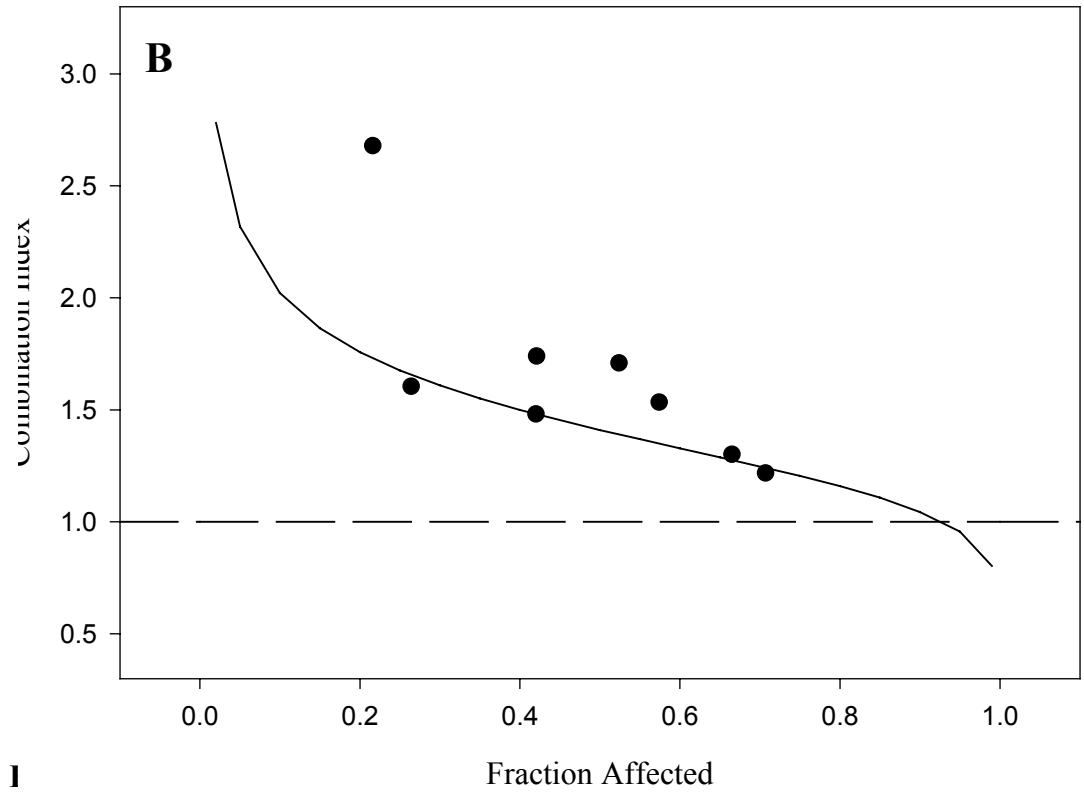
Fraction Affected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2.0
1.5
1.0
0.5
0.0
0.2
0.4
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0.8
1.0
Fraction Affected

I