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Electrophysiological findings in the Sprague–Dawley rat induced by moderate-dose carboplatin

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Abstract

Carboplatin is a second generation platinum-containing anti-tumor drug which selectively alters the micromechanical function of the inner hair cells (IHCs) of the organ of Corti in the chinchilla. Data from a recent study [Wake et al., Acta Otolaryngol. 116 (1996) 374–381], using the chinchilla model, have suggested that a moderate dose of carboplatin alters the efferent feedback loop gain of the OHCs. The present study was designed to evaluate the possible 'efferent feedback alteration mechanism' in the Sprague–Dawley rat using distortion product otoacoustic emissions (DPOAEs). A moderate dose of carboplatin (50 mg/kg body weight) was administered by a 30 min i.p. infusion. Pre- and 72-h post-treatment DPOAE and auditory brainstem response (ABR) recordings were acquired from a group of 12 rats. The animals were anesthetized with a ketamine–atropin anesthesia administered in two consecutive phases. The DPOAE responses (cubic distortion products) were recorded with four asymmetrical protocols: P1 = 60–50, P2 = 50–40, P3 = 40–30 and P4 = 30–20 dB SPL (sound pressure level), in the frequency range from 4.0 to 16 kHz. ABR responses were obtained for bipolar clicks and tone pips at the frequencies 8.0, 10.0, 20.0 and 30 kHz using stimuli in the range from 100 to 30 dB SPL. Significant ABR threshold shifts of 15 dB were observed at 30 kHz, and shifts of 10 dB at 20, 16 and 10 kHz. The comparison of pre- and post-treatment DPOAE responses did not reveal any significant changes for protocols P1, P2 and P4. Data from the P3 protocol indicated a decrease of the DPOAE amplitude. The findings from the rat model suggest that (a) moderate doses of carboplatin do not affect the efferent feedback loop OHC function and (b) the cochlear susceptibility to carboplatin across species is different, even at moderate-dose regimes.

Key words: Ototoxicity; Carboplatin; Cisplatin; Otoacoustic emissions; Distortion products; DPOAEs; ABR

1. Introduction

Carboplatin is a second-generation antitumor drug, which shows a significantly lower incidence of nephrotoxicity and ototoxicity in comparison to cisplatin (Taudy et al., 1992; Alberts, 1995; Meyer et al., 2001). Traditionally, in experimental animals the overall alteration of the hearing threshold due to a platinum compound administration has been studied by the use of auditory brainstem responses (ABRs) (Fausti et al., 1992; Ravi et al., 1995; Riggs et al., 1996; Campbell et al., 1996; Taudy et al., 1992). These measurements represent the integration of individual responses from many neural fibers, and to obtain a more detailed description of the dysfunction of cochlear micromechanics, caused by ototoxic drugs, recordings of otoacoustic emissions (OAEs) can be used (Hofstetter et al., 1997a; Hatzopoulos et al., 2002). The OAEs are considered responses of cochlear origin generated when the auditory periphery is stimulated by a click or a pure tone stimulus. Their close relationship with

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the non-linear micromechanics of the outer hair cells (OHCs) has been well established (McFadden and Plattsmier, 1984; Brownell et al., 1985; Brownell, 1990). Within this context, it can be said that the use of OAEs can provide not only a verification of the presence of toxic effects on cochlear micromechanics, but evidence regarding the progress of the ototoxicity as seen from the perspective of the OHCs.

Shera and Guinan (1999) proposed two classes of OAEs. The click-evoked OAEs (TEOAEs) are generated from reflection off pre-existing micromechanical impedance perturbations, distributed along the organ of Corti, which might include such conditions as disorganized OHC arrays (Lonsbury-Martin et al., 1988). Distortion product OAEs (DPOAEs) arise primarily from non-linear elements in the cochlea that are stimulated by the incoming traveling waves. The TEOAE responses from laboratory animals demonstrate a short bandwidth caused by the frequency limitation of the invoking stimulus, i.e. the acoustic click (Khvoles et al., 1996; Khvoles et al., 1998; Hatzopoulos et al., 1999; Sochalingam et al., 2000). Although TEOAEs are useful in a general evaluation of the cochlear function, the DPOAEs are the tool of choice to probe cochlear functionality at higher frequencies (>8 kHz).

A number of previous studies have shown that inner ear susceptibility to ototoxic drugs is dose-dependent and varies considerably across different species (Saito and Aran, 1994; Riggs et al., 1996; Hatzopoulos et al., 1999; Sochalingam et al., 2000). For carboplatin, the induced ototoxic patterns vary depending on the animal model used. Studies on chinchillas (Wake et al., 1993, 1994, 1996; Jock et al., 1996; Hofstetter et al., 1997a, 1997b; McFadden et al., 1998; Lockwood et al., 2000; Ding et al., 1999) have indicated that carboplatin-induced oxidative stress manifests first at the level of the inner hair cells (IHCs). At higher dosages of carboplatin (200 mg/kg body weight (b.w.)) the OHC population is also affected (Hofstetter et al., 1997a,b) and the induced ototoxic insult is reflected by the significant reduction of the amplitude of the DPOAEs (Hofstetter et al., 1997a). Earlier studies on carboplatin ototoxicity in the guinea pig (Saito et al., 1989, 1995; Taudy et al., 1992) have reported that high dosages of carboplatin (>150 mg/kg b.w.) destroy primarily OHCs. In the rat high dosages of carboplatin (>190 mg/kg b.w.) affect both IHCs and OHCs (Husain et al., 2001a, b).

There is evidence in the literature suggesting that at moderate dosages of carboplatin different cochlear mechanisms are at play. In a recent study by Wake et al. (1996) moderate doses of carboplatin (400 mg/M^2) were used to damage the IHCs of chinchillas. TEOAEs were not reduced but they were amplified. The authors of that study have hypothesized that the main mechanism responsible for this OAE signal alteration was the malfunction of the efferent feedback on the OHCs, caused by a malfunction of the IHCs and thus by the alteration of the afferent input to the cochlear nuclei.

This study evaluated the effects observed by Wake et al. (1996) in another animal model, the Sprague–Dawley (SD) rat. The main objective was the verification of any enhancement effects on the OHCs, after moderate carboplatin administration. We have hypothesized that these effects would be manifested as a statistically significant increase of the DPOAE amplitudes. In our evaluation of the carboplatin ototoxicity we have: (a) accessed cochlear functionality with DPOAEs which offer the possibility of testing a wider range of frequencies in comparison to the bandwidth of the TEOAE responses used in Wake et al.'s study; (b) evaluated the cochlear function at various operating points of the cochlear amplifier, using different stimulus protocols. ABRs were used as validators of the induced ototoxic effects.

2. Materials and methods

2.1. Drugs

Carboplatin used in the animal treatments was Paraplatin from Bristol Myers (10 mg/ml in normal saline), which is the product used clinically in Italy. The drug was administered to anesthetized animals, according to the protocol guidelines presented in Section 2.2, in dosages of 50 mg/kg b.w. For the animal anesthesia, we used an equal-volume solution of ketamine hydrochloride (Ketavet, Farmaceutici Gellini, Italy), xylazine (Rombun; Bayer, Italy) and atropine (Atropina Solfato; Jacopo Monico, Venice, Italy). The anesthesia was used in dosages of 1 ml/kg b.w. Each ml of anesthesia contained 33.3 mg of ketamine, 6.6 mg of xylazine and 0.16 mg of atropine. Each animal received a total of 50 mg of ketamine, 13.2 mg of xylazine and 0.24 mg of atropine/kg. The employed anesthetics resulted in a light anesthesia level, necessary to immobilize the tested animal for approximately 70 min.

2.2. Animals

Twelve male SD rats obtained from Charles River, Italy (mean weight 335 ± 30 g S.D.) were treated with carboplatin administered by an intra-peritoneal infusion (post-anesthesia) of about 30 min using a micro-pump from Harvard Apparatus. The animals were treated according to the Italian guidelines DL 116/92 with reference to EEC directive no. 86-609.

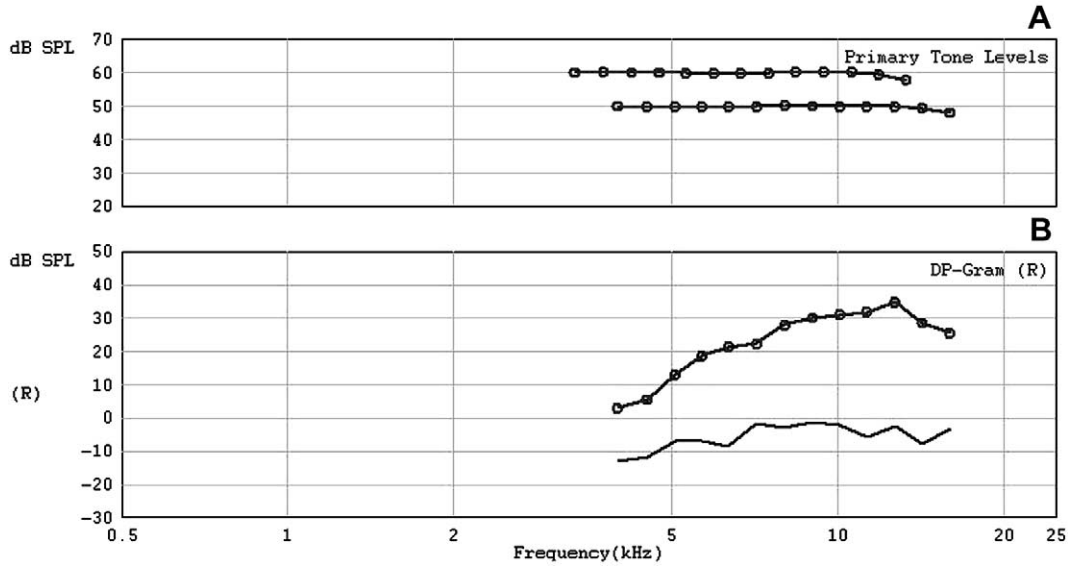

Fig. 1. DPOAEs from animal SD_4D evoked with protocol P1 (60–50 dB SPL) in the frequency range 4.0–16.0 kHz (referred to as F2). (A) Graph of the primary tone levels at the tested frequencies. When the level of each tone deviates significantly from the prefixed value (i.e. 60 or 50 dB SPL for protocol P1) the fitting of the probe is considered erroneous, and a probe reposition is necessary. (B) DP-gram at the tested frequencies. The top curve (circled line) indicates the DPOAE amplitude, while the lower line (straight) indicates the noise per tested frequency.

2.3. Electrophysiological procedures

The ABR and DPOAE responses were recorded, during anesthesia, pre-treatment and 72 h after the carboplatin administration. In the pre-treatment phase the animals (i) were anesthetized, (ii) the DPOAE and ABR responses were acquired, and (iii) the carboplatin treatment was conducted.

2.3.1. DPOAEs

The DPOAE cubic distortion responses (2F1−F2) were recorded in a sound-proof cabin by the StarKey DP-2000 apparatus. An in situ calibration was conducted on the DP-2000 transducer prior to any recording. A chirp stimulus was presented to the transducer and the resulting sound pressure levels (SPLs) were memorized. Based on these readings the DP-2000 output levels were equalized for each animal. Each animal was tested in the frequency range 4.0–16.0 kHz, with four different asymmetrical protocols coded as: P1 = 60–50, P2 = 50–40, P3 = 40–30 and P4 = 30–20 dB SPL. The frequency ratio of each protocol was set to 1.25, with a 6% resolution per octave and each frequency was sampled for 4 s. The DPOAE responses (DP-grams) presented a number of notches for frequencies above 13 kHz; therefore the analyzed data in the paper refer to F2 frequencies from 4.0 to 12.7 kHz.

In order to record the DPOAE response, the anesthetized animal was placed under a stereotaxic device. The DP-2000 probe was connected to the external meatus of the animal by a thin tube of 15 mm length and 3 mm diameter. A good seal was possible when the tube was inserted on average 5 mm into the external auditory meatus. According to the calibration data, it was not possible to attain a good probe fit at both high (>10 kHz) and low (<3 kHz) frequencies, and for this reason we have not considered DPOAE responses below 4 kHz. A typical DPOAE response from the P1 protocol (60–50) is shown in Fig. 1.

The DPOAE responses were evaluated using as a criterion a minimum signal-to-noise (S/N) ratio of 3 dB. Using the latter, a number of observations from the P4 (30–20 dB SPL) protocol were considered as missing.

2.3.2. ABRs

The ABR responses were recorded by three platinum–iridium needle electrodes, placed subdermally over the vertex (positive), the mastoid (negative) and the dorsum area (reference/ground) of the animal. The recordings were made in a sound-treated cabin whose walls and ceiling were covered by phono-absorbent material. The calibration of the sound field was done using a Bruel and Kjaer microphone (type 2209), placed 4 cm above the animal's head and facing the loudspeaker.

The ABRs were amplified 20 000 times and filtered from 20 to 5000 Hz. Each recording was the average of 500–1000 individual responses. The ABRs were generated in response to 100-μs alternated clicks and 8, 10, 16, 20 and 30 kHz tone pips (1 ms rise–fall time, 10 ms

plateau), in the range 100–30 dB SPL. The sound transducer, a Motorola tweeter (flat response ±1.5 dB from 4.0 to 35 kHz), was placed 4 cm away from the rat's ear. Threshold was based on the visibility and reproducibility of wave III, according to Bourre et al., 1999. At the minimum threshold level two recordings were acquired. During all measurements the body temperature of the animal was maintained at 38 ± 0.5°C by a rectal probe connected to a Harvard Apparatus homeothermic blanket. Ear plugs were used to occlude the contra-lateral ear in order to avoid a binaural stimulation at high stimulus intensities. Since the animals were tested and re-tested within a 72 h frame, each animal served as its own control.

2.4. Statistical analyses

To evaluate any effects on the post-treatment DPOAE responses, we have generated 95% confidence intervals for the differences in the mean DPOAE amplitudes per protocol. Both normal-theory and bootstrap BCa confidence intervals (for paired observations, i.e. pre and post) were calculated.

Bootstrapping is a method of resampling the data to estimate the underlying distribution of a statistical measure, in this case a mean difference (Efron and Tibshirani, 1993). Bootstrap intervals do not assume that the data follow any particular distribution. BCa stands for 'bias correction and acceleration', and is an adjustment which gives the intervals better statistical properties, particularly in terms of coverage probability. For more information on the application of this technique to OAE data see Hatzopoulos et al. (2002). To control the overall confidence level for the 36 computed intervals (nine tested frequencies × four protocols) at 95%, Bonferroni-adjusted confidence intervals were estimated. More details on this procedure are presented in Section A1 of the Appendix.

We have also evaluated the relationship between the ABR-induced threshold shifts and the DPOAE amplitude values per tested protocol using multiple regression models (Section A2 of the Appendix). Such a relationship is quite complex, but we have formulated the following simplistic hypothesis. Considering the traditional concept that OAEs express mainly OHC functionality and given a threshold shift indicated by the

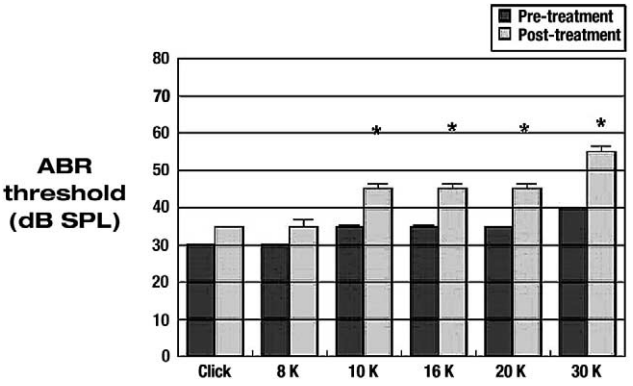

Fig. 2. ABR threshold for the pre- and post-carboplatin treatment (+1 S.E.M.). For the click responses the post-treatment responses for all animals were the same (35 dB SPL). For the 10, 16, 20 and 30 kHz stimuli, significant threshold shifts were observed, but the pre–post level differences were small. An average shift of 10 dB was observed in the frequencies 10, 16 and 20 kHz, while for the 30 kHz responses a 15 dB shift was observed. The stimulus step in the ABR acquisition system was 5 dB.

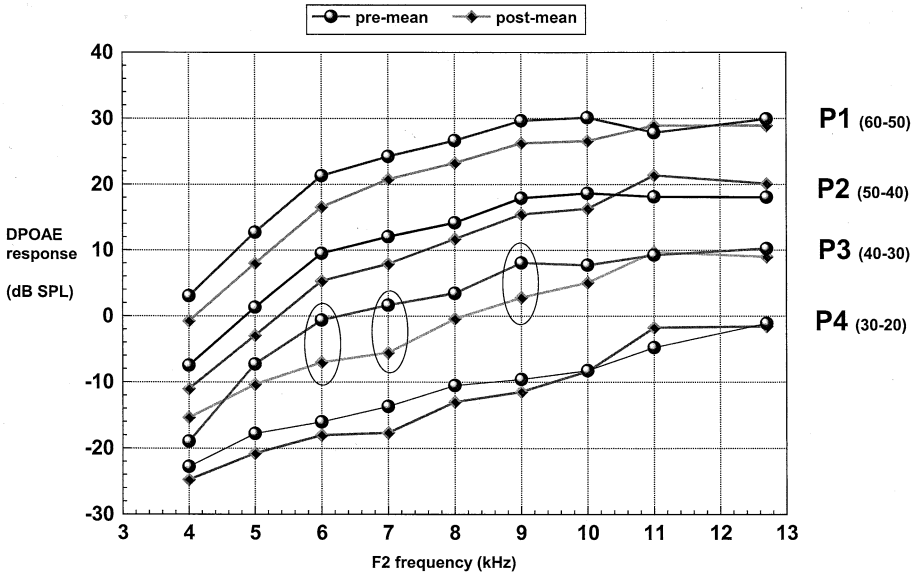

Fig. 3. Average DP-grams (pre- and post-treatment) from the four tested protocols in the frequency range 4.0–12.7 kHz. The circles indicate the three frequencies of the P3 protocol where the post-treatment DPOAE amplitudes were found statistically smaller.

ABR recordings, a poor relationship between ABR and DPOAE variables could provide additional evidence suggesting that carboplatin does not affect the efferent loop to the OHCs in the SD rat.

All analyses were implemented with a mainframe SAS package.

3. Results

A mean ABR threshold elevation of 10 dB was observed at 10, 16 and 20 kHz and a mean threshold shift of 15 dB was observed at 30 kHz, 72 h after carboplatin infusion. No significant threshold differences were observed for the click and the 8 kHz tone pip. For all tested stimuli, except the click and the 8 kHz tone pip, the post-treatment ABR responses were found significantly different from the pre-treatment responses. The data are summarized in Fig. 2. There was no significant change of weight.

Plots showing the DPOAE pre and post mean-amplitude responses (per tested protocol) are presented in Fig. 3. The 95% confidence intervals, computed from the protocols P1 (60–50), P2 (50–40) and P4 (30–20), indicated lack of any significant effects on the post-treatment DPOAE responses. These results were also validated by the Bonferroni interval estimates. A significant reduction of the DPOAE mean amplitude was observed at the tested frequencies of the P3 protocol (40–30). The post-treatment responses at the F2 frequencies 6, 7, 8, 9, 10, and 12.7 kHz were found significantly smaller than the pre-treatment responses at the same frequencies. The data were also validated by the bootstrap method, but the Bonferroni method showed that the DPOAE amplitudes were significantly smaller only at the F2 frequencies 6, 7, and 9 kHz.

4. Discussion

Platinum-containing antineoplastic drugs have generated great interest as models for ototoxic lesions. Although carboplatin, the second-generation platinum compound, is less toxic than cisplatin, the mechanism of toxicity at the cellular level seems to be mainly identical. The study evaluated the possible alteration of the suppressive efferent feedback on the OHCs, in the SD rat, treated with a moderate dose of carboplatin. Earlier data from the chinchilla (Wake et al., 1996) have suggested that moderate doses of carboplatin (400 mg/m^2) can disrupt the efferent feedback to the cochlea and alter the cochlear functionality as shown by the enhanced amplitudes of the recorded TEOAEs. The results from that study are important, because if such effects are present in other species and in humans, then using OAEs to monitor patients under carboplatin-based chemotherapy might underestimate the induced ototoxic damage. It should be noted, however, that the chinchilla model is particularly sensitive to carboplatin treatment, showing an unusual pattern of cochlear damage (IHC losses and OHCs intact) not seen in other species (Lockwood et al., 1999). The high sensitivity of the chinchilla model to cisplatin has also been noted (Hatzopoulos et al., 2002).

The results from studies evaluating the ototoxic effects of platinum-containing antineoplastic drugs depend on two main pharmacokinetic factors, namely the single-dose level and the cumulative dose. Data from a previous study on cisplatin using a guinea pig model (Ekborn et al., 2000) have suggested that the modality of administration (bolus vs. infusion) does not change the final OHC damage significantly. Based on these observations we have selected the infusion model to approximate as much as possible the currently used clinical scenarios. The experimental dosage of carboplatin determines the extent of cochlear lesions. Previous studies by Husain et al. (2001a,b) on the Wistar rat have demonstrated that severe cochlear damage (significant OHC ± IHC deterioration) results from the administration of 196 mg/kg of carboplatin. In order to obtain a minor cochlear lesion suitable for the experiment reported here, a moderate dose (50 mg/kg b.w.) of carboplatin was chosen. Administration of carboplatin at this dose level was shown to elevate the hearing threshold of SD rats at frequencies >8.0 kHz. The post-carboplatin ABR thresholds were small, suggesting minor lesions to the organ of Corti, data in agreement with observations from the guinea pig (Taudy et al., 1992). However, data from a 78 mg/kg b.w. carboplatin treatment of the chinchilla (Jock et al., 1996) suggest significant alterations of the IHC population prior to an ABR threshold elevation. These findings could suggest that the rats, guinea pigs and chinchillas have different resistance to carboplatin-induced cochlear lesions, but the small number of animals used in each study, and the different experimental protocols used, require the acquisition of additional data to elucidate this argument.

Three out of four protocols (P1, P2 and P4) indicated that there are no significant changes in the DPOAE amplitudes after moderate-dose carboplatin treatment. The observations are in agreement with data from the chinchilla studies of Jock et al. (1996) and Hofstetter et al. (1997a) for responses evoked by 60–50 dB SPL tone stimuli. The data from the P3 protocol (40–30 dB SPL) indicated a number of F2 frequencies (6–12.7 kHz) where the post-treatment responses were shown to decrease significantly. Small sample sizes (i.e. 12 animals) can introduce some biasing errors and for this reason

we have applied several methods for the statistical evaluation of the results from the P3 data set. Using the Bonferroni method, which guarantees an experiment-wise 95% confidence, the number of frequencies where decreased post-treatment DPOAE responses were observed was reduced to three (F2 = 6, 7, and 9 kHz).

It should be noted that the amplitude of the DPOAEs can be enhanced or decreased by shifting the operating point (OP) of the distortion non-linearity. Within this context, several hypotheses can be formulated following our experimental data: (1) The results from different DPOAE protocols show that in the SD rat the operating point of the distortion non-linearity is shifted (amplitudes are decreased), but does not enter a region where a change of sign is possible (where amplitudes increase); (2) it could be argued that an enhancement of the DPOAE amplitude in the chinchilla corresponds to a shift of the operating point of the distortion non-linearity into a 'sign-changing' regime. What causes that shift is currently unknown, but one can speculate on the disruption effect on the efferent feedback loop to the OHCs. To elucidate these hypotheses further, additional investigations are necessary.

The findings from the regression analysis between the ABR and the DPOAE responses (see Section A2 in the Appendix) indicated that the relationship of these two types of measurements was not significant. These analyses verify the lack of enhancement of the DPOAE amplitudes, and suggest that at this dose level of carboplatin the efferent effects in the SD rat are either very small or non-existent.

In summary, this study shows that the SD rat is less suitable for carboplatin ototoxic studies than the chinchilla in low to moderate dose regimes. At a dose level producing significant ototoxic changes in the chinchilla only borderline threshold changes were achieved in the SD rat. It is known from dose-escalation studies regarding cisplatin ototoxicity that there is a certain dose level above which electrophysiological changes can be recorded (Laurell and Engström, 1989a,b). Since carboplatin has been shown to produce more pronounced ototoxic changes in the Wistar rat at higher doses (Husain et al., 2001a, 2001b), one can expect that 50 mg/kg b.w. carboplatin is approximately the threshold dose for ototoxic effects in the SD rat. The borderline toxic effect of this dose was indirectly supported by the fact that no significant weight loss was shown. The hypothesis from Wake et al.'s group that an alteration of the afferent input (output of the IHCs) causes a disruption of the suppressive efferent mechanisms to the OHCs, was not validated in the SD rat at this dose level. In this context, our data suggest that different efferent mechanisms might suppress or enhance the activity of the OHCs of the organ of Corti across various species.

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Appendix

A1. Bonferroni intervals

For the 36 intervals (nine tested frequencies × four protocols) computed using the collected data, the Bonferroni adjustment requires that the confidence level for each interval be set at the $100\times(1-0.05/36) = 99.861\%$ level. This ensures that the probability of at least one interval failing to contain the true difference of means it is estimating is less than or equal to 0.05. Because it uses an upper bound, the Bonferroni method tends to be conservative, which means that if we are using intervals which do not contain 0 to identify significant differences in the population means, then: (a) the method finds fewer significant effects than the 5% false positive rate suggests; but (b) for these effects, we can be more confident that they are significant (i.e. the overall confidence level is likely greater than the advertized 95%).

A2. Multiple regression between DPOAE and ABR responses

To evaluate the relationship between the data, ABR values were regressed on differenced (i.e. post minus pre carboplatin treatment) DPOAE amplitude values corresponding to frequencies from 8 to 12.7 kHz. The latter was dictated by the observation that in the P3 and P4 protocols the DPOAE responses for frequencies below 7 kHz were negative or very close to zero (for both pre- and post-treatment data sets). Stepwise regression was used for model selection due to the small number of observations (in some cases, as few as six). A partial F statistic with a significance at the 0.15 level was required for regressor entry, and non-significance at the 0.15 level was required for removal.

The strongest significant relationship, having P value 0.0097 and R^2 value 0.7677 was observed for the DPOAE amplitude at 12.7 kHz (protocol P3: 40–30) and the ABR response at 16 kHz (see Table 1). It was noted that as the protocol stimulus intensity was increasing (i.e. moving from the P4 DPOAE responses to P1 responses) the number of significant relationships between ABR and DPOAE variables was decreasing and for the P1 protocol no relationship between ABR and DPOAE data was found.

Table 1
Data from the regression analyses between ABR and DPOAE responses

Protocol	ABR freq. (kHz)	DPOAE freq. (kHz)	R^2	P value
30–20 (P4)	8	none		
	10	8	0.7092	0.0354
	16	none		
	20	none		
	30	11	0.6136	0.0653
40–30 (P3)	8	12.7	0.3910	0.1332
	10	12.7	0.5774	0.0474
	16	12.7	0.7677	0.0097[a]
	20	none		
	30	11	0.6427	0.0301[b]
50–40 (P2)	8	none		
	10	none		
	16	11	0.3129	0.1495
	20	none		
	30	12.7	0.4464	0.0702
60–50 (P1)	8	None		
	10	None		
	16	None		
	20	None		
	30	None		

Column 1 indicates the DPOAE protocol (P1–P4); column 2 shows the ABR variable; column 3 shows the DPOAE variable; column 4 shows the R^2 value; column 5 shows the probability of significance ($< = 0.05$); and indicates whether the relationship between the ABR and the DPOAE variable is significant.
[a]Fit significant at the 0.01 level.
[b]Fit significant at the 0.05 level.

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