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# Tryphostin AG879, a tyrosine kinase inhibitor: prevention of transcriptional activation of the electrophile and the aromatic hydrocarbon response elements

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**Abstract**

To investigate a possible role of phosphorylation in the signal transduction pathways responsible for transcriptional regulation of drug-metabolizing enzymes, we tested seven specific tyrosine kinase inhibitors (tyrphostins) for their effects on NAD(P)H:quinone oxidoreductase-1 (NQO1) mRNA levels in mouse hepatoma Hepa-1c1c7 (Hepa-1) cells and chose to study AG879 further. The potent electrophile *tert*-butylhydroquinone (tBHQ) is known to activate *NQO1* gene transcription via the electrophile response element (EPRE). Among the tyrphostins tested, tyrphostin AG879 was unique in preventing the accumulation of tBHQ-induced NQO1 mRNA; this effect was dependent on the AG879 dose and was also sensitive to the time when AG879 was added relative to the beginning of tBHQ treatment. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (dioxin; TCDD) is known to activate *Cyp1a1* gene transcription by way of aromatic hydrocarbon response elements (AHREs). We found that AG879 also prevents, to a lesser extent, the AHRE-mediated induction of CYP1A1 and NQO1 mRNA by dioxin. Zinc or cadmium is known to activate metallothionein (*Mt1*) gene transcription via the metal response element (MRE). AG879 induced MT1 mRNA, and AG879 did not block zinc- or cadmium-induced MT1 mRNA, indicating that the effects of AG879 on NQO1 or CYP1A1 mRNA levels cannot be generalized to all transcripts. Using transient transfection of EPRE-, AHRE-, or MRE-driven luciferase reporter gene constructs in Hepa-1 cells, we showed that the inhibitory effects of AG879 occurred at the level of EPRE- and AHRE-mediated transcription, but that AG879 did not affect the MRE-driven transcriptional response. These data suggest that AG879 might inhibit an unknown tyrosine kinase(s) whose activity is essential for EPRE- and AHRE-mediated *trans*-activation of certain mammalian genes. These results also indicate that some sharing of common signal transduction pathways might exist in the regulation of genes involved in drug metabolism that also respond to oxidative stress.

*Keywords:* tyrosine kinase inhibitors; tyrphostins; electrophile response element; aromatic hydrocarbon response element; metal response element; oxidative stress; NAD(P)H:quinone oxidoreductase; cytochrome P450 1A1; Northern hybridization analysis; dioxin; luciferase reporter gene constructs; Ah receptor

## 1. Introduction

One of the most common mechanisms by which the cell communicates extracellular signals to the nucleus is through altering the phosphorylation state of specific phosphoproteins. Protein kinases are known to phosphorylate specific tyrosine, serine, and threonine residues of a protein, altering function by changing its stability, subcellular localization, or conformational accessibility [1]. These events can have important effects on gene expression, either by amplification and transduction of signals to the nucleus or by phosphorylation state-dependent translocation of transcription factors from the cytosol to the nucleus [1]. In the past decade, an improved class of pharmacological tyrosine kinase inhibitors, named tyrphostins, has been developed [2]. These compounds are designed to compete for the kinase substrate-binding site; this gives tyrphostins much greater specificity, as well as diminished cytotoxicity, relative to the more "classical" tyrosine kinase inhibitors such as staurosporine and genestein, which compete for the ATP-binding site of kinase proteins [2].

An "oxidative stress response" denotes the cell's protective adaptation to an excess of reactive oxygen intermediates or species (ROI, ROS) or oxygenated metabolites (ROMs), which can be both cytotoxic and genotoxic [*reviewed in* Refs. 3 & 4]. This complex cellular response

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results in increased levels of enzymes that participate in the synthesis of glutathione, the quenching of free radicals, the prevention of oxygen radical formation, and the repair of oxidatively damaged cellular macromolecules. The EPRE (also known as "antioxidant response element," ARE) is a DNA motif found in the upstream regulatory regions of a number of genes that are activated in response to oxidative stress [3–5]. NQO1, a 2-electron reductase that protects the cell from oxidative stress by preventing formation of 1-electron reactive oxygenated intermediates, is well characterized as one of the genes known to be transcriptionally regulated via the EPRE [4–8]. There is limited information, however, about the identity of proteins that bind to the EPRE [9–11] or the nature of events occurring at the EPRE that lead to activation of gene transcription.

Few reports have addressed the issue of phosphorylation in the study of the oxidative stress response. Realizing the importance of phosphorylation in regulating gene expression, coupled with our limited understanding of mechanisms behind EPRE- and oxidative stress-regulated transcription, we have attempted to delineate a signal transduction pathway involved in this response by screening tyrphostins. Using a particular tryphostin (AG879), our data suggest that this tyrosine kinase inhibitor blocks both EPRE- and AHRE-mediated transcription, but this inhibitor does not affect MRE-mediated transcription.

## 2. Materials and methods

### 2.1. Chemicals

The "tyrphostin inhibitor set II" was purchased from Calbiochem. Nucleotides were obtained from NEN. tBHQ was bought from Sigma. TCDD was a generous gift from the Dow Chemical Company. DNA-modifying enzymes were purchased from New England Biolabs. All other laboratory reagents and chemicals were purchased from either Sigma or Fisher and were of the highest grade available.

### 2.2. Cell culture conditions, transfections, and treatment

The mouse liver hepatoma cell line Hepa-1c1c7 was used in all experiments described herein. Cells were maintained in Dulbecco's Modified Eagle's medium (Life Technologies) supplemented with 5% fetal bovine serum (Life Technologies). Cells were grown at 37° in a humidified chamber containing 5% $CO_2$. For isolation of total RNA, cells were grown in 10-cm dishes (Becton Dickinson) in 10 mL of medium and treated with 10 $\mu$L of test compound dissolved in DMSO. For each treatment group, two dishes of cells were treated and the scraped cells were pooled. For controls, the cells were always treated with the vehicle (DMSO) alone.

For transient transfection, $2.5 \times 10^7$ cells were seeded in 5 mL of medium in a 25-$cm^2$ flask. Twelve hours after plating, a calcium phosphate precipitate of plasmid DNA (500 $\mu$L) was added. Twelve hours following the addition of precipitate, the cells were rinsed with PBS, trypsinized, and divided into 12-well plates in a volume of 1.5 mL of medium per well. Twelve hours after splitting, the cells were treated with the various inducing chemicals at the concentrations indicated. Following a 12-hr treatment, the cells were washed once in cold PBS, lysed in 100 $\mu$L of 1X reporter lysis buffer (Promega), and stored at −70° until analysis.

### 2.3. Plasmids

RNA probes for the mouse *Sod* and *Mt1* genes were synthesized from plasmids previously described [12]. The NQO1 mRNA probe was synthesized from the full-length mouse cDNA [13]. The CYP1A1 mRNA probe was synthesized from the *Stu* I fragment derived from the 3′ untranslated region of the mouse gene [14]. For transient transfection, CMV$\beta$Gal and pGL3Control (referred to in this report as *SV40luc3*) were purchased from Promega. The *Mt1min* plasmid contains the minimal metallothionein promoter only [15], cloned into pGL3Basic. The *EPREmt1* plasmid contains a single EPRE from the mouse *Gsta1* enhancer region (−754 to −714; Ref. 16), fused to the minimal mouse *Mt1*, promoter, and this was cloned into the pGL3Basic (Promega) backbone. The *AhRDtk* plasmid contains the mouse *Cyp1a1* AhRD enhancer (−1100 to −896; four AHREs) fused to the herpes simplex virus type I thymidine kinase (*tk*) minimal promoter (−79 to +53) from which the SP1-binding site was removed [17,18]. The *MREd$_5$mt1* plasmid contains a concatamer of five MREd sequences [Ref 15] from the mouse *Mt1* promoter fused to the minimal mouse *Mt1* promoter (−42 to +60).

### 2.4. RNA isolation and northern blotting

Total RNA was isolated from cells using the guanidinium thiocyanate/phenol extraction procedure [19]. Cells were lysed in guanidinium isothiocyanate/phenol/sodium acetate, the phases were broken with chloroform, and the supernatant fraction was collected and precipitated with an equal volume of isopropanol. The resulting pellet was dissolved in SDS buffer, extracted with phenol/chloroform

*Abbreviations:* EPRE, electrophile response element; AHRE, aromatic hydrocarbon response element; MRE, metal response element; *Nqo1* and NQO1, mouse NAD(P)H:quinone oxidoreductase [also called NMO1, quinone reductase, DT-diaphorase] gene and mRNA; *Cyp1a1* and CYP1A1, mouse cytochrome P450 1A1 gene and mRNA; *Mt1* and MT1, mouse metallothionein-1 gene and mRNA; *Sod*, mouse Cu,Zn-superoxide dismutase gene; *SOD*, rat Cu,Zn-superoxide dismutase gene; SOD, mouse and rat Cu,Zn-superoxide dismutase mRNA and protein; *Luc* and LUC, firefly luciferase gene and enzyme; BGAL, $\beta$-galactosidase enzyme activity; tBHQ, *tert*-butylhydroquinone; dioxin (also TCDD), 2,3,7,8-tetrahydro chlorodibenzo-*p*-dioxin; SSC, standard sodium citrate; and SET, sodium ethylenediamine tetraacetic acid (EDTA) Tris buffer.

(1:1 v/v), and RNA in the supernatant fraction was precipitated in 3 M ammonium acetate. The resulting pellet was resuspended and precipitated with 0.1 M ammonium acetate and 70% percent ethanol; this pellet was then dissolved in MilliQ-purified water.

To prepare RNA samples, we denatured 40 $\mu$g RNA in a solution of 5% deionized formamide, 5% formaldehyde, and 1X 3-(*N*-morpholino)propanesulfonic acid (MOPS) for 5 min at 70°. The sample was snap-cooled on ice, and 10 $\mu$L of RNA loading dye was added. RNA was separated on a 1% agarose (FMC Bioproducts) 1X MOPS gel. RNA was transferred to a Nytran membrane (Schleicher & Schuell). Membranes were prehybridized at 65° for 2 hr in 50 mL of prehybridization buffer (0.45 M NaCl, 90 mM Tris–HCl, pH 8.0, 6 mM EDTA, 0.1% SDS, and 2 mg/mL each of Ficoll, BSA, and polyvinylpyrolidone), and then one additional hour in 20 mL of the same solution plus yeast tRNA (100 $\mu$g/mL). Hybridization was carried out overnight at 65° in 10 mL of the same solution containing $2 \times 10^7$ counts per minute (cpm) of the RNA probe. Membranes were washed for 1 hr in 1X SSC, 0.1% SDS, and for one additional hour in 0.1X SSC, 0.1% SDS. Membranes were exposed to X-OMAT-AR autoradiography film (Kodak) overnight. Semiquantitative analysis of signal was performed using the Storm 860 Phosphorimaging system and ImageQuant 5.0 software (Molecular Dynamics).

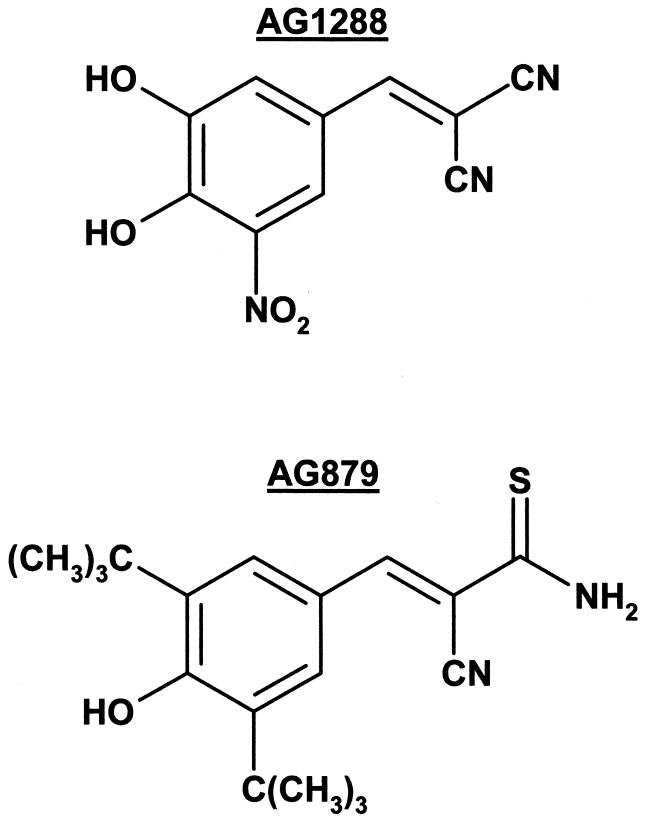


Fig. 1. Chemical structures of two tyrphostins discussed in the text. AG1288 [$\alpha$-cyano-(3,4-dihydroxy-5-nitro)cinnamonitrile]; AG879 [$\alpha$-cyano-(3,5-di-*tert*-butyl-4-hydroxy)thiocinnamide].

## 3. Results

### *3.1. Induction of NQO1 mRNA by tBHQ blocked by tyrphostin AG879*

To identify tyrphostins that had potential effects on the response to oxidative stress, we initially screened a series of tyrphostins for their effects on NQO1 mRNA accumulation. Each of the tyrphostins A1, A23, A47, A63, AG370, AG879, and AG1288 was examined at its 50% inhibitory concentration ($IC_{50}$, as indicated in the vendor's catalog), in two types of experiments. In the first experiment, Hepa-1 cells were treated with the tyrphostin at its $IC_{50}$ concentration for 3.5 hr. In the second experiment, the tyrphostin was introduced 30 min prior to a 3-hr exposure to 50 $\mu$M tBHQ, a dose that we had previously established causes maximal NQO1 mRNA induction (data not shown). Examining Northern blots of NQO1 mRNA (data not shown), we found two of the seven tyrphostins to be of particular interest.

Tyrphostin AG1288 was the only one of the seven that induced NQO1 mRNA. Examination of the chemical structure of AG1288 (Fig. 1), however, revealed that it has an *ortho*-phenol moiety which can non-enzymically form a quinone and, hence, is likely to undergo redox cycling. Because we would not be able to distinguish easily the effects of redox cycling from the effects on the protein phosphorylation state during *Nqo1* induction, we decided not to study this compound further. On the other hand, tyrphostin AG879 (Fig. 1), at its $IC_{50}$ of 10 $\mu$M, prevented NQO1 mRNA induction by tBHQ. Because inhibition of this induction response is likely to reflect modification of the phosphorylation states of proteins involved in signal transduction, we chose to characterize further this effect of AG879.

### *3.2. Dose- and time-dependent effects of AG879 on NQO1 mRNA Induction*

Fig. 2 depicts the dose–response inhibition by AG879 on tBHQ-induced NQO1 mRNA levels. We chose not to use doses of AG879 that exceeded the $IC_{50}$ of 10 $\mu$M in order not to saturate the target kinase; such saturation could potentially lead to less specific inhibition of additional unknown kinases. Fig. 2 shows a proportional dose–response inhibition by AG879 between the range of 0.3 and 10 $\mu$M, with the 10-$\mu$M dose completely inhibiting tBHQ-induced NQO1 mRNA, and doses of 0.3 $\mu$M or lower having a negligible effect. SOD mRNA, used here as a loading control and to demonstrate RNA integrity, did not change in response to AG879 treatment.

What is the effect of the time of addition of AG879 relative to the initiation of tBHQ treatment? AG879 was

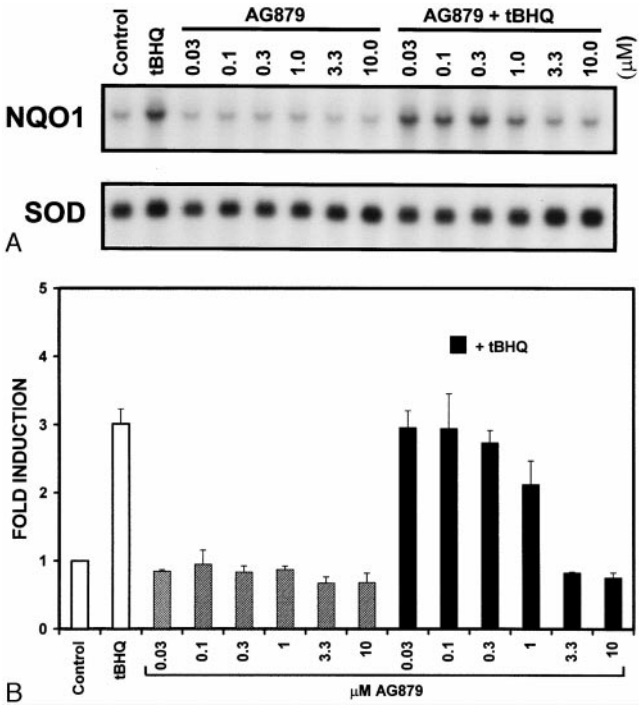


Fig. 2. Dose-dependent effects of AG879 on NQO1 mRNA in mouse Hepa-1 cell cultures. (A) Representative Northern blot of an experiment repeated twice. The cultures were pretreated with AG879, at doses between 0.03 and 10 $\mu$M, for 30 min, following which there was a 6-hr incubation in the presence or absence of tBHQ (50 $\mu$M). Control cells were treated with vehicle alone. All cells were harvested after 6.5 hr. SOD is shown as a loading control. (B) Histogram of experimental data in Fig. 2A. The arbitrary units represent the semiquantitative densitometry of the NQO1 mRNA bands, divided by the SOD mRNA bands for each lane, and data are expressed normalized to the density in the control lane. The bars and brackets denote the means and standard deviations from duplicate samples in two independent experiments.

added to the cell cultures at several time points, before and after initiation of tBHQ exposure (Fig. 3). Whereas tBHQ-mediated NQO1 mRNA induction was strong when AG879 was added late, increases in mRNA were not seen when AG879 was added between 6 hr before, and 1 hr after, the beginning of tBHQ treatment.

### *3.3. Inhibition of tBHQ activation of the EPRE by AG879*

Northern blots (Figs. 2 and 3) cannot distinguish between transcriptional activation and mRNA stabilization. Because NQO1 induction by tBHQ is known to be mediated through the action of an EPRE [4–9], we posed the question as to whether AG879 might inhibit transcriptional activation through this DNA motif. tBHQ induced an EPRE-driven luciferase reporter construct about 3-fold; this response was blocked when AG879 was added simultaneously with tBHQ, with a dose-dependent response between 10 and 0.3 $\mu$M (Fig. 4). On the other hand, AG879 treatment did not affect the basal activity of the EPRE-driven reporter gene, nor did it affect the basal activity of the minimal *Mt1* promoter-driven, or the strong simian virus 40 promoter-driven, control luciferase expression vectors (Fig. 4).

### *3.4. TCDD-induced CYP1A1 and NQO1 induction prevented by AG879*

Dioxin activates transcription of several genes through the AHRE motif [4,17,20]. Are the inhibitory effects of AG879 on EPRE-mediated induction also seen with AHRE-

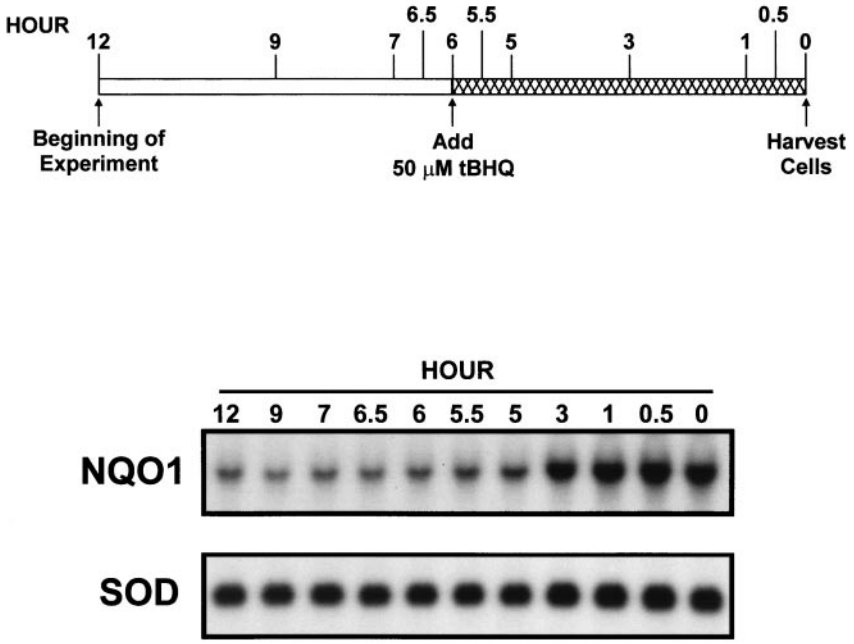


Fig. 3. Time-dependent effects of AG879 on NQO1 mRNA. (Top) Time scale of the experiment, in which 10 $\mu$M AG879 was added as early as 12 hr before tBHQ and as late as 6 hr after the addition of tBHQ, the latter time point being the moment of harvesting the cells. (Bottom) Northern hybridization analysis of the experiment, with time points of AG879 as indicated in the time scale (top).

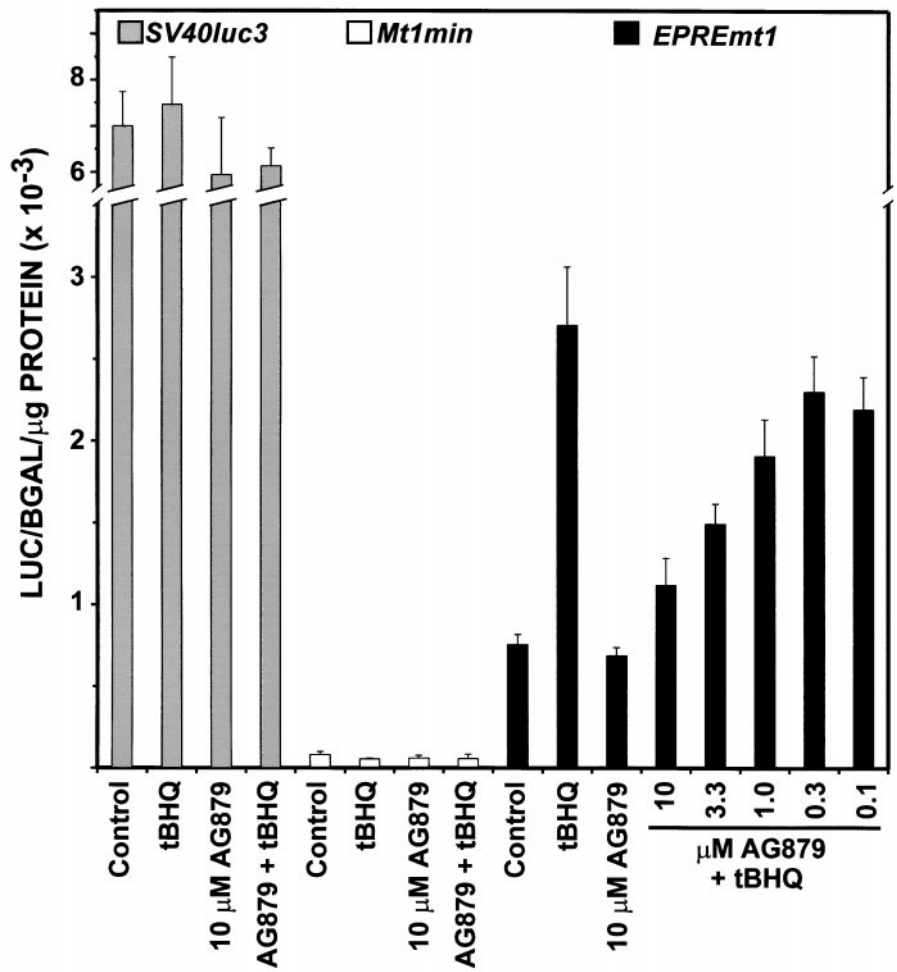


Fig. 4. Inhibition of EPRE activity by AG879. Treatment with 50 $\mu$M tBHQ, with or without AG879 at the indicated $\mu$M concentrations or vehicle alone (control), was carried out for 12 hr. Units are expressed as luciferase (LUC) luminescence units per BGAL units per $\mu$g protein, Bars and brackets denote the means and standard deviations (N = 3) from the same transfection experiment for the control plasmids *SV40luc3* and *Mt1min*, and means of three determinations from each of two transfections for the plasmid *EPREmt1*.

mediated induction? To answer this question, we treated Hepa-1 cells with dioxin (Fig. 5). AG879 almost completely blocked CYP1A1 mRNA induction when the TCDD dose was 0.1 nM; with larger doses of TCDD, however, CYP1A1 mRNA induction was less inhibited by AG879. In contrast, AG879 completely blocked NQO1 mRNA accumulation in response to 0.1, 1.0, or 10 nM TCDD. AG879 (10 $\mu$M) had a negligible effect on the basal CYP1A1 or NQO1 mRNA levels.

### *3.5. Less potent inhibition of dioxin activation of the AHRE by AG879*

As we did with the EPRE construct (Fig. 4), we sought to demonstrate that this AG879 effect was transcriptional rather than posttranscriptional by using a reporter gene construct (Fig. 6). Whereas dioxin induced the *AhRDtk* plasmid about 30-fold, AG879 added concomitantly at a dose of 10 $\mu$M prevented this induction by approximately 50%. On the other hand, AG879 at a dose of 1.0 $\mu$M was ineffective.

### *3.6. MT1 mRNA induction by AG879*

If AG879 inhibits EPRE- and AHRE-mediated expression nonspecifically, would AG879 have a similar effect on yet another gene expression pathway? We chose to study MT1 induction as a pathway that is distinct from the dioxin-inducible [*Ah*] gene battery [4], but which does respond to oxidative stress [3]. Both zinc and cadmium are well known as inducers of *Mt1* transcription via MRE-mediated transactivation [15,21,22]. Fig. 7 shows that zinc or cadmium caused a 30- to 40-fold increase in MT1 mRNA. Interestingly, AG879 alone, at concentrations of 10 or 3 $\mu$M, produced about a 20-fold increase, whereas 1.0 $\mu$M AG879 gave a 4-fold increase. When cadmium or zinc was added concomitantly with AG879, MT mRNA accumulation was slightly (but not statistically significantly) higher than the effect by metal treatment alone.

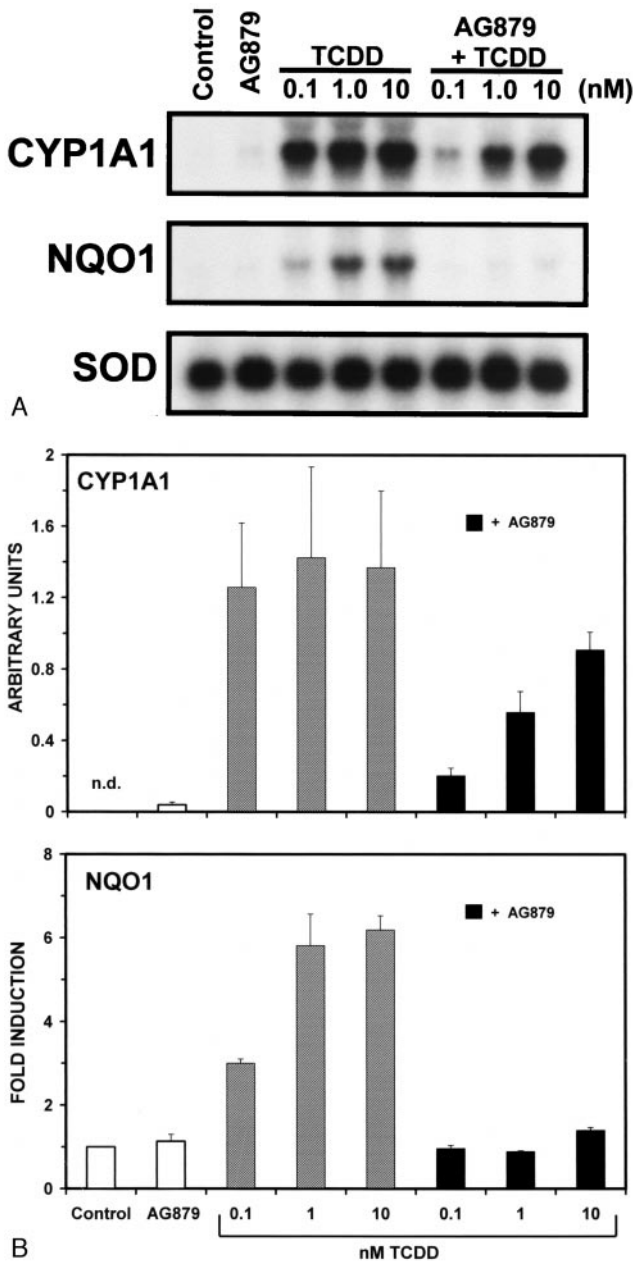


Fig. 5. Prevention of TCDD-induced CYP1A1 and NQO1 mRNA accumulation by AG879. (A) Representative Northern blot of an experiment repeated twice. Three different concentrations of dioxin were used; AG879, when used, was 10 $\mu$M. Control cells were treated with vehicle only. All cells were harvested 6 hr after treatment. SOD is shown as a loading control. (B) Histogram of experimental data in Fig. 5A. The arbitrary units represent the semiquantitative densitometry of the CYP1A1 or NQO1 mRNA bands, divided by the SOD mRNA bands for each lane, and data are expressed normalized to the density in the control lane. The bars and brackets denote the means and standard deviations from duplicate samples in two independent experiments. n.d., not detected.

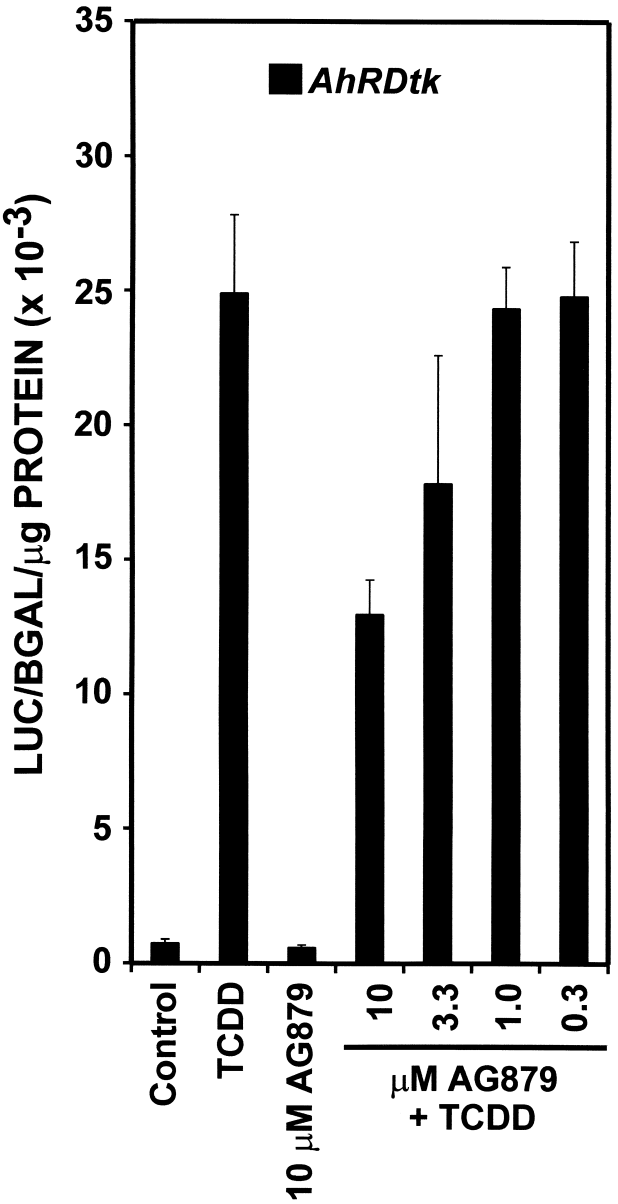


Fig. 6. Inhibition of AHRE activity by AG879. Treatment with 1 nM dioxin, with or without AG879 at the indicated $\mu$M concentrations or vehicle alone (control), was carried out for 12 hr. Units are expressed as luciferase (LUC) luminescence units per BGAL units per $\mu$g protein. Bars and brackets denote the means and standard deviations from three determinations on each of two transfections for the plasmid *EPREmt1*. Data from the control plasmids *SV40luc3* and *Mtmin* (not illustrated) were similar to those shown in Fig. 4.

### *3.7. AG879 effects on MT1 mRNA accumulation: no mediation by MRE*

Again, as in Figs. 4 and 6, we sought to distinguish between transcriptional activation and mRNA stabilization by using a reporter gene construct (Fig. 8). Whereas the inducer zinc was found to increase MRE *trans*-activation almost 4-fold, AG879 had no effect at doses of 1 to 10 $\mu$M, nor was there any effect of AG879 when added simultaneously with zinc.

## 4. Discussion

In this report, we have shown that the tyrosine kinase inhibitor AG879 blocks tBHQ induction of NQO1 mRNA.

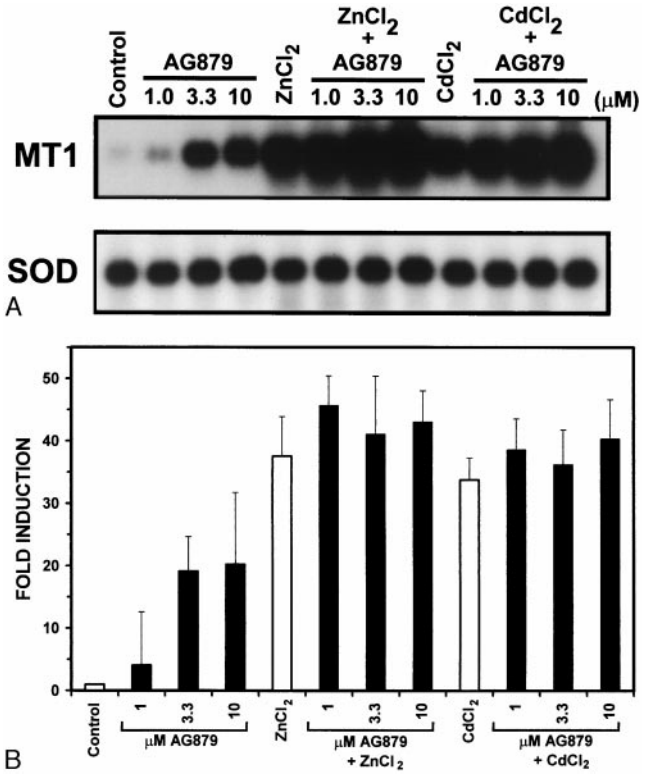


Fig. 7. Induction of MT1 mRNA by AG879. (A) Representative Northern blot of an experiment repeated twice. Three different concentrations (1–10 $\mu$M) of AG879 were studied; 100 $\mu$M $ZnCl_2$ or 25 $\mu$M $CdCl_2$ was used as the inducer where indicated. Control cells were treated with vehicle only. All cells were harvested after 6 hr of treatment. SOD is shown as a loading control. (B) Histogram of experimental data in Fig. 7A. The arbitrary units represent the semiquantitative densitometry of the MT1 mRNA bands, divided by the SOD mRNA bands for each lane, and data are expressed normalized to the density in the control lane. The bars and brackets denote the means and standard deviations from duplicate samples in two independent experiments.

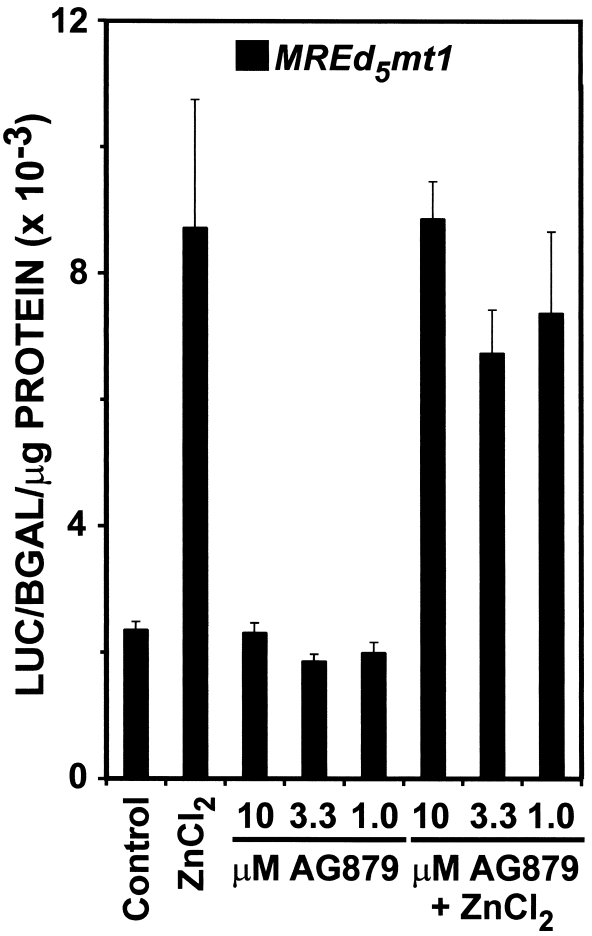


Fig. 8. No effect of AG879 on MRE *trans*-activation. Treatment with AG879 alone, 100 $\mu$M $ZnCl_2$ or 25 $\mu$M $CdCl_2$, with or without varying $\mu$M concentrations of AG879, or vehicle alone (control) was carried out for 12 hr. Activity is expressed as luciferase (LUC) luminescence units per BGAL units per $\mu$g protein. Bars and brackets denote the means and standard deviations from three determinations on each of two transfections for the plasmid *MRE$d_5$mtl*. Data from the control plasmids *SV40luc3* and *Mtmin* (not illustrated) were similar to those shown in Fig. 4.

We began our investigation by screening a battery of tyrphostins for their effects on inducible *Nqo1* gene transcription. Only two of the tryphostins that we screened had consistent effects on NQO1 mRNA levels. Whereas the induction seen using AG1288 could easily be explained by its quinone structure, AG879 was chosen for further study because the observed effect was likely to be a result of the inhibition of a signal transduction pathway induced by oxidative stress and culminating in *Nqo1 trans*-activation.

The EPRE is known to be necessary and sufficient to mediate the oxidative stress response in several cell types [4–8, 23] and is of critical importance in the transcriptional induction of the mammalian *Nqo1* gene [6–8]. Using transient transfection of an EPRE-driven reporter gene construct, we demonstrated that AG879 does indeed block tBHQ-induced EPRE *trans*-activation. These data strongly support the likelihood that AG879 stops NQO1 mRNA accumulation by inhibiting its transcriptional induction. This conclusion is further supported by the time-course of AG879 addition (Fig. 3). Treating cells with AG879 for up to 6 hr before the addition of tBHQ had an inhibitory effect on NQO1 mRNA accumulation; this result would not be expected if AG879 were to destabilize the mRNA. Adding AG879, at or near the beginning of induction, was effective in preventing NQO1 mRNA accumulation. Also, the addition of AG879-3 or more hr after the triggering of induction with tBHQ was ineffective at preventing mRNA accumulation. These observations collectively indicate that AG879 can act quickly—and in a sustained manner—to prevent *trans*-activation mediated by an unknown kinase(s), but AG879 has no effect on mRNA that has already been transcribed.

The above-described effects of AG879 on tBHQ-induced NQO1 were also seen on TCDD-induced CYP1A1 and NQO1 mRNA (Fig. 5). It is interesting, however, that doses of AG879 that totally suppressed tBHQ-induced NQO1 did not completely inhibit dioxin-mediated induction of CYP1A1 mRNA. AG879 was much more effective at inhibiting CYP1A1 mRNA accumulation in cells treated with

0.1 nM TCDD. In similar experiments using an AHRE reporter construct (Fig. 6), 1 nM TCDD was used as the inducing dose, based on our Northern blot findings (Fig. 5) that this dose induces both CYP1A1 and NQO1 mRNA maximally. AG879 at 10 $\mu$M inhibited by about 50% the induction of the *AhRDtk* construct, whereas lower doses of AG879 were less effective. As with *Nqo1* induction, these results suggest that AG879 inhibits *Cyp1a1* induction at the level of transcription. It has been demonstrated that transcriptional activation of *Cyp1a1* is inhibited by the tyrosine kinase inhibitors staurosporine and genestein [24,25]. As is also true with this study, the tyrosine kinase(s) responsible for inhibition were not identified in these reports. Our results also suggest that AG879 may be less potent in preventing *Cyp1a1* induction than *Nqo1* induction. Another possible explanation is that the unknown kinase, which is inhibited by AG879, might induce transcription only to some upper limit, but that certain genes, when induced maximally, rely on this kinase for only a certain percentage of the signal transduction necessary to induce transcription fully.

This speculation is difficult to reconcile, given our observation that AG879 completely inhibited TCDD-induced NQO1 induction over a dose range of 0.1 to 10 nM TCDD (Fig. 5). Complex cross-talk is observed, however, among genes in the [*Ah*] battery [4]. Oxidative stress, which activates the EPRE, has been demonstrated to down-regulate *Cyp1a1* and *Cyp1a2* expression [26,27], and a negative response element in the *Cyp1a1* promoter has been described [28–32]. Several reports suggest a possible interaction between components of the AHRE and EPRE motifs [33–35]. Hence, it is possible that multiple signal transduction pathways, each requiring regulation of phosphorylation states, are involved in the response to monofunctional and bifunctional inducers [8] of [*Ah*] battery genes. It is likely that overlapping and redundant signal transduction cascades have evolved to respond to more than one cellular stimulus in similar, yet distinct, manners. The kinase(s) inhibited by AG879 may be able to regulate some genes more specifically (or completely) than others, or may regulate certain genes to a different extent based on the perceived cellular condition (cell division, differentiation, apoptosis, stress response). A less plausible possibility is that AG879 might prevent transport of tBHQ or TCDD into the cell, and that larger doses of inducer would override this effect.

After seeing similar effects on both EPRE- and AHRE-mediated activities, we had considered that AG879 might be acting as a general inhibitor of gene transcription. Several observations, however, support the specificity of action of AG879. (a) G879, at all doses tested, had no effect on the basal levels of several mRNAs (Figs. 2, 3, 5, and 7), which rules against AG879 being a general suppressor of transcription. (b) AG879 showed no toxic effects at any dose tested, as judged by trypan blue exclusion in the cells; transcription is often nonspecifically lowered in the presence of generalized toxicity. (c) AG879 had a negligible effect on basal levels of *SV40luc3* and *Mt1min* reporter activity (Fig. 4) and AG879 also had no effect on basal levels of the *EPREmt1*, *AhRDtk*, and *MRE$d_5$mt1* plasmids (Figs. 4, 6, and 8). (d) Transcriptional induction through the EPRE is apparently mediated through NRF2 while forming a heterodimer with a member of the small MAF family or Jun [10,11, *reviewed in* Ref. 3], and activation through the AHRE is mediated by AHR/ARNT heterodimers [36–38]. As noted above, there is considerable cross-talk among these factors. On the other hand, transcriptional induction of *Mt1* is mediated in large part through the MRE as a result of the activation of metal response element binding transcription factor 1, MTF-1 [15,21,22]. Neither *Mt1* induction by metals nor MTF-1-driven transcription is inhibited by AG879.

During the preparation of this manuscript, Yoo and coworkers [39] reported AHR-mediated regulation of rat SOD mRNA levels. If this is also true in the mouse Hepa-1 cells used herein, then SOD as a loading control would not be valid. We therefore re-evaluated this by examining the variation in SOD mRNA following dioxin treatment. Each RNA sample was quantified by absorbance at 260 nm, and 5 $\mu$g per lane was loaded based on this measurement. Furthermore, all samples were stained with ethidium bromide to assess integrity and equal loading of RNA. Based on these criteria, all RNA demonstrated equal loading; specific mRNAs not affected by an experimental treatment would therefore be expected to vary marginally based on differences in loading and blotting efficiency. We found no significant difference in SOD mRNA between untreated and dioxin-treated cells, as assessed by phosphorimaging analysis (data not shown). Since the criterion for loading was based on total RNA and not SOD mRNA, we must conclude that SOD does not change as a result of treatment. Species-specific differences in the promoter region of the mouse versus rat *SOD* gene, or the use of different cell lines, might explain the discrepancies between the report of Loo *et al.* [39] and the present study. In addition, we also found no statistically significant effect of AG879 on the basal expression levels of NQO1 or CYP1A1 mRNA (Figs. 2, 3, and 5).

We had considered that the observed effects of AG879 might be attributed to mechanisms besides inhibition of tyrosine kinases. Although no data are presented to demonstrate unequivocally that the observed effects of AG879 are a result of inhibiting a tyrosine kinase, several lines of reasoning support this conclusion. AG879 is one of a family of compounds that are characterized foremost for their common ability to inhibit tyrosine phosphorylation, and AG879 has been demonstrated in several reports to have this effect. We had considered that AG879 might prevent NQO1 induction by tBHQ by acting as a scavenger of reactive oxygen. Experiments with AG879 using *in vitro* radical-generating assays (negative data not shown) ruled out this possibility. These experiments with distinct models of inducible gene transcription strongly suggest that AG879's mechanism of action is not through radical scavenging,

because neither the inhibition of CYP1A1 mRNA induction through the AHRE nor the induction of MT1 mRNA would be expected to occur via a mechanism involving radical scavenging.

A model of *trans*-activation through the EPRE is beginning to emerge in the literature, although conflicting results from several researchers complicate the picture. Several studies implicate the NRF2 transcription factor as part of the complex assembled at this element [10,11,40–42; reviewed in Ref. 3]. Studies also suggest the involvement of the related NRF1 protein, as well as members of the small MAF family, and possibly JunB [10, 43; reviewed in Ref. 3]. Whereas no reports to date have addressed the phosphorylation state of NRF or MAF family proteins in the context of oxidative stress-induced transactivation, it is tempting to speculate that AG879 might affect the phosphorylation of one or more of these proteins.

Unfortunately, we cannot assign the observed AG879 effects to a specific kinase at this time. Although tyrphostins are much more specific than kinase inhibitors that are designed to compete for the ATP-binding site of proteins, tyrphostins are still subject to the same limitations as all pharmacologic agents [2]. Therefore, there might be nonspecific interactions with several unknown cellular components, and most likely more than one kinase is inhibited by AG879. Published reports on the specific effects of AG879 are limited [44–49]. Potentially germane to the observations in this report, in PC12 cells stimulated with nerve growth factor, AG879 inhibits pp140c-*trk* tyrosine phosphorylation, but does not affect tyrosine phosphorylation of epidermal growth factor or platelet-derived growth factor receptors [44]. In addition to stating that AG879 prevents PI-3 kinase activity and phosphorylation of PLC-γl, this report also describes inhibition of nerve growth factor-induced c-*fos* proto-oncogene activity and mitogen-activated protein (MAP) kinase activity [44]. Because AP-1 transcription family proteins have been implicated in the response to oxidative stress [7,10,50–52], this is a possible explanation of the effects of AG879 on EPRE-mediated induction shown in the present study. Indeed, two recent reports [53,54] have addressed the involvement of MAP kinase pathways in *trans*-activation through the EPRE. In particular and germane to this report, the use of a specific MAPK kinase inhibitor, PD98059, abolished extracellular signal-related kinase 2 (ERK2) activation and impaired the induction of NQO1 activity [54]. Although NQO1 mRNA levels were not examined, EPRE reporter activity was partially inhibited by PD98059 [54], suggesting that this inhibitor is acting partially at the level of transcription. These experiments also used higher doses of inhibitor (50 μM) to achieve only partial inhibition of EPRE-mediated reporter activity [54], whereas we found that a 10-μM dose of AG879 completely blocks EPRE-mediated reporter activity, suggesting a higher specificity of AG879 than of PD98059 for the pathway in question.

Because PLC-γ1 activity has been implicated in the induction of AHR-inducible genes [4], this could also explain the effect of AG879 on AHRE-mediated inducibility. Also, because protein kinase C has been described as necessary for the activation of the AHR [25,55–57], inhibition by AG879 of PLC-γl phosphorylation—leading to a decrease in protein kinase C activity—is consistent with observations in the present study. Also possibly relevant to the observations in this report, AG879 has been described as an inhibitor of HER2/Neu [46]. Also known as erbB2, HER2/Neu is a transmembrane glycoprotein with tyrosine kinase activity [58]. AG879 inhibits the androgen-dependent *trans*-activation of the prostate-specific androgen gene, which is thought to be activated through a MAP kinase pathway [49]. These data, combined with findings in the present study, suggest that signaling necessary for EPRE and AHRE activation might be initiated in a membrane protein at the cell's surface and may require MAP kinase activity.

In this report, we have shown that AG879 blocks EPRE- and AHRE-mediated *trans*-activation, but not MRE-mediated activity. These data indicate that tyrphostins are valuable tools to study gene regulation. These initial experiments will allow our laboratory to focus on the study of signal transduction pathways implicated in EPRE- and AHRE-mediated *trans*-activation. These experiments also underscore the importance of phosphorylation in the cellular response to exogenous oxidants and halogenated aromatic hydrocarbons. Because transcriptional up-regulation of genes in the [*Ah*] gene battery, such as *Nqo1*, is considered a mechanism by which cells resist chemotherapy [59], identification of kinase(s) involved in the transcriptional regulation of these genes could provide the basis for the design of drugs used in adjunct chemotherapy.

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