DEVELOPMENT OF *IN VITRO* BIOLOGICAL AND BIOCHEMICAL ASSAY PLATFORM FOR RATIONAL DRUG DESIGN

Thesis

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INTRODUCTION

Signal transduction and tumour therapy

The morbidity and mortality caused by cancers are one of the biggest challenges for the Medicine. With the intense research over the last decade, the breakthrough, such as the antibiotics for the infectious diseases, is waiting to be discovered. According to the conception of signal-transduction therapy, distinct signalling molecules play roles in some important aspects of such diseases' development and progression as cancers, inflammatory or autoimmune diseases and cell death related neurodegenerative diseases, and are promising targets of the modern drug design and support new perspectives for the therapy. As the most important transductional mechanisms are phosphorylation processes, the new research conception aimed to inhibit protein kinases. Currantly, new drug candidates target receptor tyrosine kinases are under intense research and many of them are in clinical development.

Az EGF receptor tyrosine kinase as tumour therapeutic target

The epidermal growth factor receptor (EGFR) is a member of the ErbB (HER) receptor family. Abnormalities in functions of these receptors and their ligands are associated with all key features of cancer development and growth, including autonomous cell growth, invasion, angiogenic potential, and growth of distant metastases. Overexpression is one of the most common mechanisms leading to overactivation of the signaling pathway. Substantial evidence shows strong relationship between overexpression of EGFR and HER3 and the development of lung, colon and breast cancer (50-70%). Similarly, overexpression of HER2 and HER4 is found in 30% and 50% of primer breast cancer, respectively. HER4 is also overexpressed in 22% of primer colon cancer. Overexpression of EGFR correlates with poor outcome in several tumor types. (Normanno et al. 2003, Abd El-Rehim et al. 2004).

Recently, interest has been raised in small EGFR mutations in the catalytic domain, that underlie the sensitivity to some anilinoquinazoline inhibitors (e.g. gefitinib or erlotinib) in NSCLC (non-small cell lung cancer) patients. Discovery of this correlation was a dramatic breakthrough in NSCLC treatment that is the leading cause of cancer death in

Hungary and treatment with conventional cytotoxic agents remains suboptimal. The most fequent activating mutations in NSCLCs were in-frame deletions in the exon 19, removing amino acids 747 through 750 (delE746–A750 and delL747–P753insS) and a pointmutation in exon 21 (L858R) that are account for 85% of all mutations (Sharma et al. 2007, Pao et al 2004, Kosaka et al. 2004). One of the most interesting perspectives in terms of development of 2nd generation inhibitors is selection of novel drug-candidates on the basis of enhanced specificity towards mutated as opposed to wild-type EGFR.

Not all EGFR kinase mutations are associated with hypersensitivity to gefitinib and erlotinib. A single secondary mutation in EGFR exon 20, T790M, arised in a subset of EGFR-mutant tumours that recure after an initial response to gefitinib or erlotinib and caused resistance to these kinase inhibitors. This mutation in EGFR is present in the same allele that carries the activation mutations (Kobayashi et al. 2005). The T790M mutation is structurally analogous to the mutated gatekeeper residue T315I in Bcr-Abl, T670I in c-KIT and T674I in PDGFRα that weaken the interaction of inhibitors, such as imatinib with the kinase. New drugs targeting these mutations are in clinical development. Similarly, the design of additional EGFR inhibitors targeting the kinase domain with T790M mutation is necessary.

The resistance mutations are relatively rare, and tumours that fail to respond to gefitinib or elotinib despite the presence of an EGFR mutation might have sustained other genetic lesions associated with resistance. c-MET gene amplification has been proposed as a mechanism of acquired resistance in NSCLC (Bean et al. 2007). While overexpression of IGFR, Her3, activated Her2 or VEGF has been linked to primary resistance to gefitinib. Activation of downstream effectors in Ras/MAPK and PI-3K/Akt pathways (e.g. K-Ras, Akt) or defect of PTEN have also been proposed to have a role in mediating resistance to EGFR inhibitors (Ahrendt et al. 2001, Anderson et al 2001, Festucci1 et al. 2005). Similarly, inhibition of mTOR serine/treonine kinase sensitizes rezistant tumours to gefitinib (Rao et al. 2005). New strategies to overcome such primary or acquired resistance are based on development of dual inhibitors or inhibitors targeted downstream effectors.

Novel agents target EGFR

Specific EGFR inhibition approaches are currently among the most promising and the most advanced in the clinical setting. The strategies use monoclonal antibodies, tyrosine kinase inhibitors, antisense oligonucleotides blocking EGFR synthesis, and antibody-based immunconjugates.

The tyrosine kinase inhibitors are synthetic, mainly quinazoline-derived, low molecular weight molecules that interact with the intracellular tyrosine kinase domain. Gefitinib (Iressa) and erlotinib (Tarceva) are the most advanced of their class and have received approval for the treatment of refractive NSCLC, where they significantly prolonged overall survival in patients with EGFR activating mutation. (Han et al. 2005, Kaneda et al. 2004). Despite, drug resistance in NSCLC patients is a critical therapeutic problem and development of alternative strategies targeting EGFR signal transduction is necessary. Dual targeting approches are one of these strategies. The other is development of a new inhibitor class (EKB-569, ZD6474, GW-572016 and CI-1033) that covalently bind to ATP-binding site in the kinase catalytic domain and promise more effective inhibition against EGFR with resistance mutation (Bonomi P. 2003).

Our research conception

In the frame of our research project we intend to apply a novel approach of rational drug design starting with the generation of a focused molecule library which is pre-screened by computational methods against the pharmacophore models derived from the validated target molecules and their agonists/antagonists. The possible leads filtered out this way will be the starting structures for the synthesis of focused combinatorial libraries. Data from biological assays of related to these compounds will be used in the refinement of the original pharmacophore model and in the generation of new virtual leads. Experimental data from physical-chemical and biological characterisation of optimised lead structures will be used also for the fine tuning of our rationally designed pharmacophore models and generation of new virtual leads for further drug development. On the base of the structure-activity relationship analysis, we intend to produce small molecular tyrosine kinase inhibitor compounds around ATP analogue pharmacophor structures.

Efficacy characterization of the molecule libraries are performed in *in vitro* biochemical assay and tumour cell lines with cellproliferation assays (MTT, Methylene Blue)

AIMS

The aim of our research program in collaboration with the chemical research group is to develop new ATP-analogues that inhibit EGFR receptor tyrosine kinase for therapeutic interventions. By so doing we could contribute to further enhance of tumour therapy and reducing of clinically relevant side-effects. Our most interesting perspectives in terms of development of 2nd generation inhibitors is selection of novel drug-candidates against EGFR with rezistance mutations

Set up biochemical assay for efficacy characterisation of inhibitory compounds

Our aim was to established a simple, rapid and cost-effective ELISA-based enzymatic bioassay for screening a large set of EGFR inhibitors.

Producing recombinant EGFR

To meet the needs of high amount EGFR enzyme arising in the biochemical assay technology, we were planning to establish a Baculovirus expression vector system. We produced recombinant wild type EGFR kinase and its four mutant forms, the two most frequent activation mutation forms (L858R pointmutation and delL747-P753insS 18-bases deletion) and their combination with T790M rezistance mutation, using site-directed mutagenesis PCR.

Screening

We characterised the efficacy of the compounds synthetized by our chemical partnergroup with our ELISA-based biochemical assay and paralelly in cell proliferation assays (Methylene blue and MTT test). Data from biological screening are used in the refinement of the original pharmacophore model and in the optimization of lead molecules.

We plan to establish an *in vitro* tumour model system of lung cancer to investigate the efficacy of EGFR inhibitors in lung cancer cell lines with different EGFR status.

METHODS

Compounds

Compounds synthetized or re-synthetized by the chemical partner group, gefitinib, erlotinib, CI-1033, EKB-569, PD153035, AG1478

EGFR enzymes

Commercial wild type EGFR purified from A431 cells (Sigma) or commercial wild type recombinant EGFR (Proqinase), recombinant wild type, deL747-P753insS deletion mutant, L858R pontmutant and L858R/T790M duble mutant EGFR kinase domain produced in our laboratory.

Cell lines

A431, H1650 (delL747-P753insS EGFR mutation, loss of PTEN), H1975 (L858R and T790M EGFR mutation), H1666 (Raf mutation), H358 (Ras mutation), Sf9

Experimental methods

Protein tyrosine kinase assay – ELISA-based biochemical method for efficacy characterisation of inhibitory compounds

Cellproliferation assays: MTT and Methylene Blue methods for investigation antiproliferative effect of inhibitory compounds on A431 cells

Baculovirus expression vector system – for production of wild type and mutant EGFR kinase domain in Sf9 cells

Restriction fragmentlength analysis and sequencing – for analysis of vector constructions

Protein izolation – glutathione agarose bead-based purification of the fusion protein with GST-tag

SDS-PAGE and Western blot – for analysis of eluated protein

Statistic analysis – Student t-test, SW, MSR

RESULTS

Biochemical assay set-up

We established a simple, rapid and cost-effective ELISA-based enzymatic bioassay to characterise the efficacy of a large number of compounds. We adapted the protocol of PTK-101 kit by Sigma to our reagents. We optimised the conditions of the detection- and tirozin kinase enzymatic reaction, determined the kinetic parameters of the enzymatic reaction and selected the optimal reference inhibitors for positive controls in the screening protocol. We examined the signal window (SW) to check that there is sufficient separation between the maximum signals (1U EGFR purified from A431cells) and the background signals (no enzyme control). On the basis of acceptance criteria, our assay is well optimized to conduct compound screening (SW=2.79).

Kinetic studies to determine v_{max} and K_m values were performed using various amounts of Poly(Glu, Tyr)$_{1:4}$ substrate (1.6-250 μg/ml), different incubation time (30-180 min) and two different sources of EGFR enzyme. Enzymes showed normal Michaelin-Menten kinetics with the following values: K_m=1.064 μg/ml, v_{max}=0.0045 for 1 U of EGFR purified from A431 cells (Sigma); and K_m=23.9 μg/ml, v_{max}=0.0074 for 50 ng of recombinant EGFR (ProQinase). According to these data, we calculated the optimal Poly(Glu, Tyr)$_{1:4}$ substrate concentration in 10 fold of the K_m values which results in an initial reaction velocity where the assay is in the linear range.

We compared inhibitory efficacy of three reference inhibitors (PD153035, gefitinib and Genistein) on the EGFR purified from A431 cells (Sigma) (David W. Fry et al, 1994; Wakeling et al, 2002; Tetsu Akiyama et al, 1987). PD153035 and gefitinib were shown to be the most effective compounds reaching 90% of inhibiton in concentration range of 50-0.08 μM ($p<0.05$) with the IC_{50} values of 2.68 and 3.46 μM, respectively. Both drugs can be used as positive control in the screening protocol. Inhibitory efficacy of these two compounds was investigated in concentrations of 50-0.00064 μM for the recombinant EGFR (ProQinase) as well. Both inhibitors had higher efficacy on this latter enzyme with IC_{50} values of 0.071 and 0.18 μM, respectively, and these values were more comparable with the literature data.

Assay reproducibility was examined in 4 independent measurements of inhibitory efficacy (T/C%) of gefitinib at 50 μM, and on the basis of the MSR values the assay was stable over time (MSR=4.6).

Production of recombinant EGFR

A Baculovirus expression vector system is used to express wild type, and mutant EGFR with delL747-P753insS deletion, L858R pontmutation and their combination with T790M substitution in Sf9 insect cells. Mutations were generated by site-directed mutagenesis PCR. Nucleotide sequences were checked with sequencing analysis. The protein products were GST-fusion EGFRs containing the EGFR kinase domain and purified with glutathione agarose beads. Western blot analysis and SDS-PAGE were performed to confirm the purity and homogeneity of the products. Catalytic activity of all lots was determined in our enzymatic bioassay. Reproducibillity between the lots was also investigated in the cases of 50 ng wild type and L858R mutant EGFR and on the basis of 4 lots (MSR_{vad}=5.21; MSR_{L858R}=1.47) we considered well reproducible.

Screening compound in biochemical assay

We determined EGFR inhibitory efficacy of 74 compounds in our optimized biochemical assay using wild type recombinant protein by ProQinase in concentration of 50 ng/well, and PD153035 as positive control in 50-2-0.4 μM concentrations. Eleven of the compounds reached the 50% of inhibitory efficacy in the investigated concentration range of 50-10-2-0.4 μM. We investigated inhibitory activity of 22 additional kinase inhibitors on wild type and 3 mutant forms (delL747-P753insS, L858R and L858R/T790M) of EGFR produced in our laboratory. Seven compounds were shown to be effective on at least one of the enzyme types, reaching 50% of inhibitory efficacy. One of them blocked the mutated form (T790M) responsible for the gefitinib/erlotinib resistance in NSCLC patients (IC_{50}=32.83 μM)..

Biochemical assay-based selectivity profiling of 6 clinically relevant kinase inhibitors on mutant forms of EGFR

Six drugs with confirmed tyrosine kinase inhibitory efficacy (gefitinib, erlotinib, PD153035, AG1478, CI-1033 és EKB-569) were investigated on wild type and 3 mutant forms of EGFR (delL747-P753insS, L858R and L858R/T790M EGFR) produced in our laboratory, in biochemical assay (Bonomi P. 2003, Bridges et al. 1996, Partik et al. 1999). In this study all compounds inhibited the wild type and activating mutant forms of EGFR. The reistance mutant (T790M) EGFR was sensitive only to the CI-1033 and EKB-569 irreversible inhibitors with the IC_{50} values of 3.96 and 882.6 nM, respectively; $p<0,01$). CI-1033 had the highest inhibitory effect on all EGFR forms with the IC_{50} of 0.017 nM for wild type EGFR. Inhibitory efficacy of all the compounds was higher on the activating mutant enzymes than the wild type form and of the two activating mutations, deletion mutation was more sensitive to the compounds. IC_{50} values of gefitinib were 0.35 nM for deletion mutant EGFR and 1.67 nM for L858R mutant EGFR. In the case of erlotinib these were 9.42 nM and 19.58 nM and for PD153035 these were 0.18 nM and 0.49 nM, respectively. The highest difference in the IC_{50} values between the two activating mutations was detected in the case of gefitinib (4.8-fold). Since the IC_{50} values of the two clinical compounds (CI-1033 and EKB-569) for the activating mutant enzymes were much lower than the lowest concentration measured in the assay, we couldn't calculate these values. In contrast to the results above, AG1478 had higher inhibitory effect on the L858R mutant EGFR (IC_{50}=1.77 nM) than on the deletion mutant one (IC_{50}=4.78 nM) ($p<0.001$) and inhibited the wild type EGFR with a lower IC_{50} value (IC_{50}=0,79 nM) than gefitinib (IC_{50}=24.71 nM), erlotinib (IC_{50}=369.5 nM), EKB-569 (IC_{50}=6.76 nM) and PD153035 (IC_{50}=5.31 nM) ($p<0.001$).

To reveal the correlations between the observed differences in the inhibitory effects and the protein-inhibitor interactions, we docked the inhibitors into the putative binding site of the wild-type EGFR. The strongest binding was found for CI-1033, while erlotinib showed the weakest binding. Our docking scores were in good agreement with the $\ln(IC_{50})$ values.

Antiproliferative efficacy characterization in cell proliferation assay

Paralelly and prior to set-up biochemical assay, antiproliferative efficacy on A431 cell line of 143 compounds were determined in MTT and Methylene Blue methods after 6 and 48 hour-treatment. Of these compounds, 58 were efficiently inhibited the cellproliferation after 48 hours and 6 were considered to be toxic since causing cell death after 6 hours.

We also established a lung cancer model system in which we can extend our cell-based screening system to lung cancer cell lines expressing different forms of EGFR and harbouring other alterations (H1975, H1650, H1666, H358). In this system we investigated the antiproliferative efficacy of gefitnib in concentration range of 20-0,002 μM with MTT method. Different handling protocols were used before the treatment (stavation, normal culturing, and treatment with 100 ng/ml of EGF) and inhibitory efficacy was compared in these different cell lines. Our results showed that handling protocols mentioned above strongly influenced the IC_{50} values for gefitinib in H1975 and H1650 cell lines. Starving the H1975 cells sensitized them to the drug (IC_{50}=6.54μM), while EGF treatment resulted in resistance of these cells. In contrast, gefitinib had higher inhibitory efficacy on H1650 after treatment with EGF (IC_{50}=2.06μM) and inhibited cellproliferation of these cells at higher IC_{50} values in starvation procedure (IC_{50}=44.79 μM) ($p<0.001$).

DISCUSSION

Biochemical assay set-up

The major focus of our research program by applying a novel approach of rational drug design is to develop new ATP-analogue compounds that inhibit EGFR receptor tyrosine kinase. In this study we characterised inhibitory efficacy of compounds synthesized by the chemical partnergroup in biochemical assay. For this purpose we established and optimized a simple, rapid and costeffective ELISA-based biochemical assay for large number of compounds. The assay reproducibility is a crucial financial and timing issue in computed modelling assisted rational drug design, because in many cases, previously

analyzed and characterized compound libraries have to be re-synthesized for repeated screening.

During the assay set-up we followed and adapted the protocol of PTK-101 kit by Sigma to our reagents. We determined the kinetic parameters of the enzyme reaction and found differences in the v_{max} and K_m for the Poly(Glu, Tyr)$_{1:4}$ synthetic substrate with two different commercial enzymes. K_m value was 20-fold lower in the case of EGFR purified from A431 cells than in the case of recombinant EGFR, which can be explaind with the different affinity of the enzymes to the substrate due to the different protein structure. To assume the assay linearity where less than 10% of the substrate has been converted to product, substrate concentration for the screening protocol was determined in 10-fold of the K_m value. In the case of the recombinant EGFR used in the subsequent measurements was 240 μg/ml. Of three investigated reference inhibitor, the inhibitory efficacy of both PD153035 and gefitinib reached 90% in the investigated concentration range, so both compounds can be used as positive control in the screening protocol. Comparative study of these inhibitors showed significant difference between the IC_{50} values for EGFR purified from A431 (Sigma) and recombinant EGFR (Proqinase) ($p<0.001$). IC_{50} values of both drugs were lower for the recombinant enzyme. Our data provide evidence that purity/source of the enzyme as well as the substrates used for detecting phosphorylated tyrosine residues or suboptimal reaction conditions strongly influence the sensitivity and reproducibility of the assay, which explain inter-laboratory differences. Since the IC_{50} values of the reference inhibitors for the recombinant EGFR were closer to the literature data (David W. Fry et al, 1994; Wakeling et al, 2002) and the higher K_m value for this enzyme allows the perform of measuremenst between larger absorbance range with keeping the assay in the linear portion, finally, we adapte the method to recombinat enzyme. This also allows generation of mutations in the EGFR kinase domain in our laboratory. Investigation of the assay variability and the separation of the maximum signals and the background signals indicated that our biochemical assay is well optimized and reproducible for efficacy characterization.

To meet the needs of high amount of the EGFR enzyme in the biochemical assay, we established and insect cell-based Baculovirus expression vector system to produce recombinant EGFR molecules with or without mutation. We generated two activating mutations (L858R and DelL747-P753insS) in the kinase domain and their combination

with the resistance mutation (T790M) by PCR-based site directed mutagenesis. On the basis of the MSR values for 4 lots of wild type and L858R mutant EGFR, we considered the reproducibility between the lots appropriate.

Screening compounds in biochemical assay

In our biochemical assay we tested 96 kinase inhibitory compounds. Twenty two of them were investigated on the EGFR mutant forms as well. Sixteen compounds were shown to be effective on the wild type EGFR, 7 inhibited the catalytic activity of one of the two activating mutant forms and one compound acted on the resistance mutant form. This compound can serve as a starting structure to develop new, most successful drugs against this mutant enzyme.

Biochemical assay-based selectivity profiling of 6 clinically relevant kinase inhibitors on mutant forms of EGFR

In this study, we compared the inhibitory efficacy of gefitinib and erlotinib and additional four investigational compounds (CI-1033, EKB-569, PD153035 and AG1478) on different mutant forms of EGFR using a simple ELISA-based biochemical assay developed in our laboratory. This study allowed estimating the predictive power of the assay in respect of their clinical efficacy. Our results confirmed the difference in sensitivity of the four status of EGFR to all the six compounds. Gefitinib has higher inhibitory effect on both wild-type and activating mutant forms of EGFR than erlotinib ($p<0.001$). The enzyme was more sensitive to both drugs above in the presence of the activating mutations that corresponded to the most of the literature data (Mukohara et al. 2005). Both gefitinib and erlotinib produced more pronounced inhibitory efficacy to the deletion mutant form of EGFR than the point mutant one ($p<0.001$), and these results are in good agreement with the clinical observations confirming the predictive power of this simple ELISA-based kinase assay (Mitsudomi et al. 2005, Riely et al. 2006). In clinical trials, gefitinib appears to be more effective in patients with exon 19 deletion than in patients with other mutations, such as L858R as our results of a clinical study also showed (Pinter et al. 2008). The potential clinical application of our biochemical evidence of the extremely selective potency of both erlotinib and gefitinib on mutant

EGFR proteins could mean a dose reduction in EGFR-mutant NSCLC patients suffering from serious side-effects while keeping the benefit of the treatment.

Among all the compounds investigated in this study, CI-1033 was the most effective inhibitor of all types of EGFR. Wild-type EGFR was inhibited at concentrations of this drug that are 1000-fold lower than that of gefitinib. Both CI-1033 and EKB-569 inhibited the resistance mutant form of EGFR, however, CI-1033 was much more effective than EKB-569 ($p<0.01$). Neither PD153035 nor AG1478 inhibited the resistance mutant form of EGFR, but showed dramatic inhibitory effect on the other forms of the receptor. EGFR mutations were more sensitive to In contrast to the other compounds, AG1478 was observed to have higher inhibitory effect on point mutant than deletion mutant EGFR and lower concentration of this tyrphostin was necessary to inhibit wild-type EGFR than those that are needed for gefitinib, erlotinib, EKB-569 or PD153035 ($p<0.001$).

To reveal the correlations between the observed differences in the inhibitory effects and the protein-inhibitor interactions, we docked the inhibitors into the putative binding site of the wild-type EGFR. High correlation between the docking scores and the $\ln(IC_{50})$ values shows that ligand-receptor complexes predicted by FlexX program are in good agreement with the inhibitory data, and furthermore, it confirmes that our recombinant enzyme-based biochemical assay is a good screening model for selecting effective compounds. Additional studies are required to determine the same correlations in the case of the mutant EGFRs.

Our results of comparative biochemical screening of existing and novel compounds may provide the scientific reality for an optimized therapeutic application of various EGFR inhibitors and it may also provide the basis to find new template structures for the development of next-generation drugs for patients with resistance to the first generation EGFR-targeted therapeutics.

Screening compounsd in cellproliferation assay

Paralelly and before biochemical assay set-up, 143 compounds were tested on A431 cells with two independent methods (MTT and Methylene Blue test). Fifty eight of them had inhibitory effect on the cells after 48 hours and 6 compounds caused cell death in 6 hours that were considered to be toxic. Inhibitory data from both biochemical and cell-

based screening assays will be used in the refinement of the original pharmacophore model and in the optimization of virtual lead molecules.

Besides our biochemical assay platform in which we can test compounds syntetized by the chemical partner group on different mutant forms of EGFR, we established a lung cancer model system involved lung cancer cell lines expressing different status of EGFR. We are planning to extend our cellproliferation assay system to investigate EGFR inhibitors in these cells at least in cases where compounds proved in biochemical assay to be promising against some EGFR form.

Investigation of the antiproliferative effect of gefitinib in this model system confirmed that pre-treatment (starving, treatment with EGF) of the cells before treating with the inhibitor could modulate the sensitivity of the cells to gefitinib. This observation can explain the contraversal data from studies using cell lines. Althought on the basis of the lower IC_{50} values from cellproliferation assay-s with H358 and H1666 showed that these cell lines were more sensitive to gefitinib than H1650 and H1975, considering their Ras and Raf mutant status, these inhibitory efficacy of gefitinib can be due to the higher proliferation rate of these cell lines (data not shown). Different behaviour of H1650 cells after starvation and EGF treatment can be explained with the oncogenic stress mechanism, while sensitivity to gefitnib of H1975 cells after starvation might be connected to the off-target efficacy of gefitinib. On the basis of our results, all 4 cell lines are considered to be resistant to gefitinib, which is confirmed by the expanded literature data in the last few years connected to revealing the genetic status of these cell lines (Pao et al. 2005, Tracy et al. 2004, Mukohara et al. 2005, Janmaat et al. 2006, Sordella et al. 2004). Overall, this model system allows antiproliferative efficacy characterization of compounds in rezistance tumour cells to EGFR targeted drugs, revealing mechanisms behind the responds and finding downstream targets to override this rezistance.

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