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Discovery of potent and selective PKC-θ inhibitors

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Abstract—An uHTS campaign was performed to identify selective inhibitors of PKC-θ. Initial triaging of the hit set based on selectivity and historical analysis led to the identification of 2,4-diamino-5-nitropyrimidines as potent and selective PKC-θ inhibitors. A homology model and initial SAR is presented demonstrating that a 2-arylalkylamino substituent in conjunction with suitable 4-diamino substituent are essential for achieving selectivity over many kinases. Additional hit to lead profiling is presented on selected compounds.

Protein kinase C theta (PKC-θ) is a member of the protein kinase C serine/threonine kinase family that has recently been shown to play a critical role in T cell signaling. The novel PKCs of which PKC-θ is a member (nPKC-δ,ε,η,θ) require DAG and PS but are calcium independent. PKC-θ, unlike most other PKCs, has restricted expression, found only in T cells and skeletal muscle.[1] The murine knockout phenotype was specific to defective NF-kB signaling in mature T cells.[2] TCR-mediated NF-kB activation was absent from PKC theta −/− mature T cells but intact in thymocytes. NF-kB activation by IL1 and TNF alpha was equivalent to wild-type animals. T cell levels were normal. However, T cell activation was severely depressed, as determined by IL2 secretion and T cell proliferation. Upon T cell activation, PKC theta is the isoform that translocates upon antigenic stimulation of TCR.[3] The phenotype of PKC-θ knockout mice is specific to T cell activation and is a calcium independent pathway. The knockout phenotype indicates that a PKC-θ inhibitor would be an immunosuppressive agent since it renders mature T cells unable to proliferate and release IL2. Unlike described immunosuppressive agents, a PKC-θ inhibitor would not alter T cell development. Autoimmune diseases, such as psoriasis, and complications of transplants are characterized by pathogenic responses as a result of inappropriate T cell activation. Present treatment includes cyclosporine, FK506, and steroids that reduce disease severity by reducing T cell activation. There is an unmet medical need for an immunosuppressive drug with a safer side-effect profile. This potential led to the development of an uHTS screen in hopes of identifying novel and selective small molecule inhibitors of PKC-θ. The screen and the hit to lead process which followed resulted in the discovery of a potent and selective

Keywords: PKC-θ; Kinase; Inhibitor; Hit to lead.
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series of PKC-θ inhibitors. The process of triaging the hits and the initial SAR studies will be the focus of this report.

The uHTS screen yielded a dose responsive hit set with activities that ranged from low nanomolar to high micromolar. The compound classes in the hit set were known to have members with cross reactivity to other kinases. Analysis of the hit set comparing historical data of the various classes in a panel of kinases indicated possible class related cross reactivity with VEGFR1, LYN, IR, and SYK. As these kinases have the potential for undesirable side effects in a PKC-θ inhibitor, selectivity was the primary driving factor in the initial triaging of the hit set. PKC-α and PKA counterscreens were performed on all hits with minimum of 10× selectivity required. Two classes, diaminopyrimidine (**1**) and indolinones (**2**), emerged as the focus based on potency, selectivity in the counterscreens, and available SAR.

A representative set of these compounds from exisiting analogs covering the major SAR elements of the 2 series and spanning a potency range from low nM to 1 μM were tested against IR, LYN, SYK, and VEGFR1 to determine if either series offered SAR that indicated selectivity could be achieved (Figs. 1 and 2).[4] From these data the pyrimidine class (highlighted with green tabs) had a trend toward selectivity with one compound (**3**) clearly showing very good selectivity.[5] In contrast the indolinones active for PKC-θ (no tabs) appeared to have no exploitable SAR for achieving selectivity.[6] We therefore focused our attention on developing the pyrimidine class into viable leads with good selectivity, drug like properties, and cellular activity.

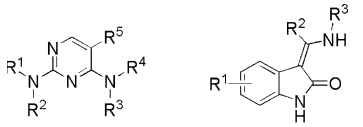


Figure 1. Two structural classes of interest from primary uHTS screen.

The pyrimidines were synthesized as shown in Scheme 1. The 2,4-dichloro-5-nitropyrimidine was treated with potassium thiocynanate in AcOH which reacts selectively at the 4-position and allows for clean differentiation of the 2- versus 4-positions.[7] Displacement of 2-chloro with arylalkylamines proceeded smoothly followed by displacement of the thiocyanate with an appropriate amine gave the desired compounds.[8] An excess of diamines was necessary to avoid diaddition or alternatively diamines could be mono-protected and used in an addition-deprotection sequence.[5]

Initial homology modeling of PKC-θ based on CDK2 followed by docking of **3** suggested that the pyrimidine binds to the hinge region at the ATP binding site via a 2-point binding of the 2-amino substituent and nitrogen of the pyrimidine at the 1 position with Leu461. Another prediction was the key interaction of the distal amine of the 4-substituent with Asp508. The model suggested that modifications of the 2-substituent may lead to additional hydrogen bond interactions with Tyr460 as well as affect selectivity by steric interactions with the region near Leu386 proximal to the glycine-rich loop and the specificity surface near Tyr468 (Fig. 3).[9]

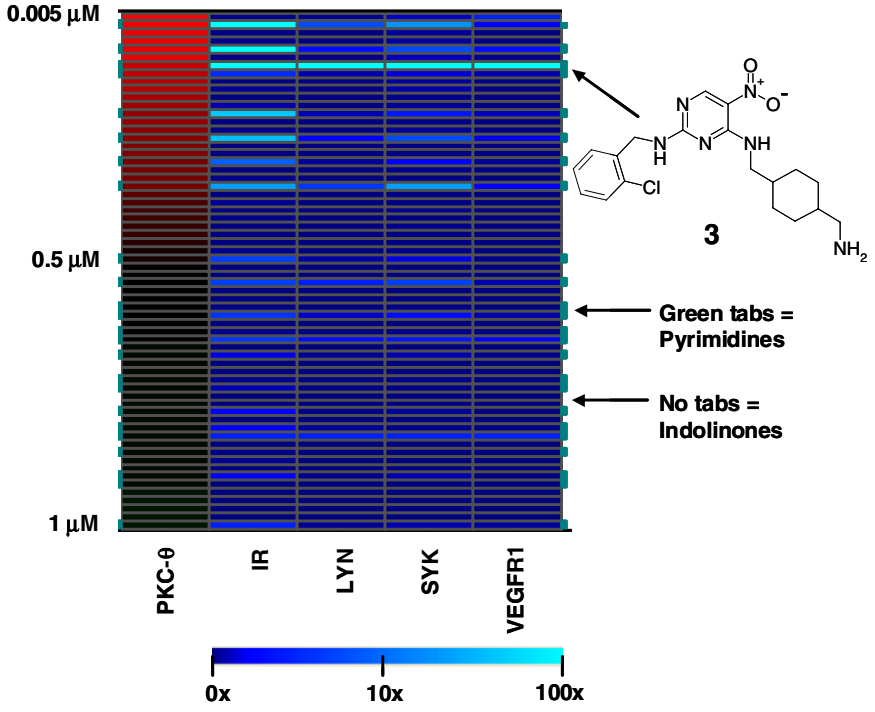
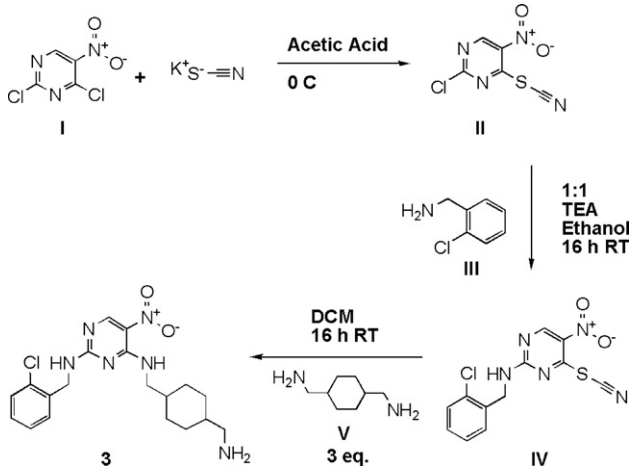


Figure 2. Heat map of selectivity.



Scheme 1. General synthetic method.

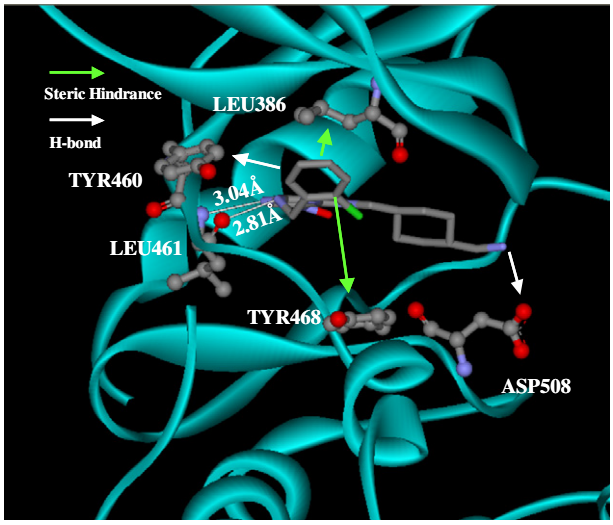
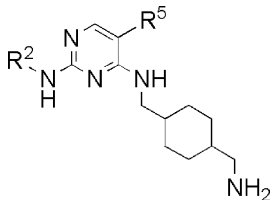


Figure 3. Homology model of PKC-θ kinase domain bound with 3.

The pyrimidine hits from the uHTS campaign had either 5-nitro or 5-trifluoromethyl substituents. The ~10-fold increase in potency in the nitro series (Table 1) led us to focus on the 5-nitro series for our initial SAR surveys at the 2- and 4-positions. The evaluation of the data in Figure 2 showed a trend for the 2-benzylamino derivatives, and especially **3**, to be more selective than the 2-anilino derivatives for PKC-θ however the 2-benzylamino series was comparatively under represented.

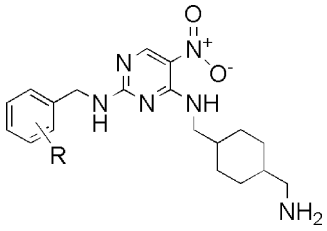
The data from the initial hits, as well as the homology model, suggested that the 2-position would render exploitable SAR. Table 2 shows the results of our early exploration of the 2-position.[10] There was a preference for *ortho* over *meta* substitution but both were beneficial compared to the unsubstituted benzyl (**3**, **8**–**18**). Substitution at the *para* position led to neutral or detrimental effects as the size of the substituent was increased, but also led to decreased selectivity (vide infra). The variations in potency based on the *ortho*-substituent were affected less by electronics than by sterics. Compare compounds **3**, **11**, **14**, and **17** where electronic nature of the substituent was varied with compounds **20**, **22**, **23**, and **24** where the steric demands are more dominant. Disubstitution was permitted and appeared to be additive with dichloro substituents in the 2,3- and

Table 1. Preference for the 5-nitro series



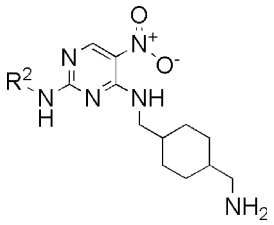
Compound	R^2	R^5	PKC-θ Inhibition IC_{50} (μM)
4	3,5-ClPh	NO_2	0.68
5	3,5-ClPh	CF_3	4.20
6	3-ClPh	NO_2	0.06
7	3-ClPh	CF_3	0.64

Table 2. 2-Position SAR



Compound	R	PKC-θ Inhibition IC_{50} (μM)
8	H	0.50
3	*o*-Cl	0.046
9	*m*-Cl	0.085
10	*p*-Cl	0.17
11	*o*-F	0.048
12	*m*-F	0.083
13	*p*-F	0.21
14	*o*-OCH_3	0.017
15	*m*- OCH_3	0.21
16	*p*- OCH_3	12
17	*o*-CH_3	0.034
18	*m*- CH_3	0.039
19	*o*-CF_3	0.012
20	*o*-OCF_3	0.018
21	*o*-CHF_2	0.015
22	*o*-SCF_3	0.006
23	*o*-SCH_3	0.005
24	*o*-Br	0.005
25	*o*-Ph	0.039
26	*o*-NO_2	0.011
27	2,3-diF	0.022
28	2,4-diF	0.10
29	2,5-diF	0.035
30	2,6-diF	0.042
31	2,3-diCl	0.008
32	2,4-diCl	0.027
33	2,5-diCl	0.007
34	2-CH_3-3-Cl	0.046
35	2,3-diCH_3	0.013
36	2,5-diCH_3	0.91
37	3,5-diCl	0.40
38	3,5-diOCH_3	1.5

Table 3. Linker variations

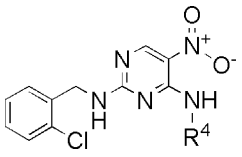


Compound	R^2	PKC-θ Inhibition IC_{50} (μM)
39	2-Br-Bz	2.2
40	$PhC(CH_3)_2$–	2.5
41	(*S*)-$PhCH(CH_3)$–	3.7
42	(*R*)-$PhCH(CH_3)$–	5.7
43	(*S*)-Indan-1-yl	4.4
44	(*R*)-Indan-1-yl	0.46
45	Indan-2-yl	0.031
46	$Ph(CH_2)_2$–	0.094
47	$Ph(CH_2)_3$–	0.27
48	$Ph(CH_2)_4$–	0.090
49	2-CH_3-$Ph(CH_2)_2$–	0.18
50	2-F-$Ph(CH_2)_2$–	0.13
51	3-F-$Ph(CH_2)_2$–	0.054
52	4-F-$Ph(CH_2)_2$–	0.077

2,5-disubstitution patterns (**31** and **33**) as contrasted with the difluoro analogs **27** and **29**. However there were size constraints and the dimethyl analog **36** showed decreased activity relative to the parent **8**. Comparatively, 3,5-disubstitution led to weaker analogs (**37** and **38**). Table 3 highlights SAR studies of the linker at the 2-position. Amide analog **39** displayed significantly reduced potency compared to **24**. Mono- or dimethylation of the methylene of the benzyl group was also detrimental to potency (**40**–**42**). Interestingly, **44** showed that the detrimental effects could be partially alleviated by ring constraining the molecule. This may be accounted for by the substitution on the aryl ring, increased linker length or restricted rotation leading to preferential orientation of the substituent. Orientation appears to be at least partially contributing as the optical antipode **43** had no increase in activity relative to **41**. The linker length did not appear to be a strong contributor to potency as the phenethyl, phenpropyl, and phenbutyl analogs all had similar values (**46**–**48**). The ring constrained analog **45** had a significant boost in potency reinforcing a preferred orientation versus the effect of substitution on the aryl moiety (compare to **49**). The *ortho* substitution on the aryl may be less preferred than *meta* in the phenethyl series (**50**–**52**).

From the model as well as SAR of the uHTS hits we expected the diamine at the 4-position would be important for activity. The role of the terminal amine in **3** was confirmed by deleting it as in **53** which resulted in loss of activity (Table 4). The spacing of the diamine was critical as was seen with analogs **62**–**64**. Restricted rotation by incorporation of a ring improved potency as seen with **3** and **59**. However, the more rigid and planar phenyl analogs **56** and **60** were significantly less potent than the saturated versions. There was no stereochemical preference for the 1,4-cyclohexylbis(methylamine)

Table 4. 4-Position SAR



Compound	R^4	PKC-θ Inhibition IC_{50} (μM)
53	Cyclohexylmethyl	>10
54	*cis*-4-(H_2NCH_2)-Cyclohexylmethyl	0.018
55	*trans*-4-(H_2NCH_2)-Cyclohexylmethyl	0.029
56	4-(H_2NCH_2)-Phenylmethyl	0.16
57	4-($(CH_3)_2NCH_2$)-Cyclohexylmethyl	0.054
58	4-($AcNHCH_2$)-Cyclohexylmethyl	>10
59	3-(H_2NCH_2)-Cyclohexylmethyl	0.003
60	3-(H_2NCH_2)-Phenylmethyl	0.20
61	Piperdin-4-ylmethyl	0.090
62	$H_2N(CH_2)_3$–	>10
63	$H_2N(CH_2)_5$–	0.45
64	$H_2N(CH_2)_7$–	0.11

substituent (**54** and **55**). The basicity of the amine was important as dimethyl analog **57** was tolerated. However, acylation resulted in the inactive analog **58**.

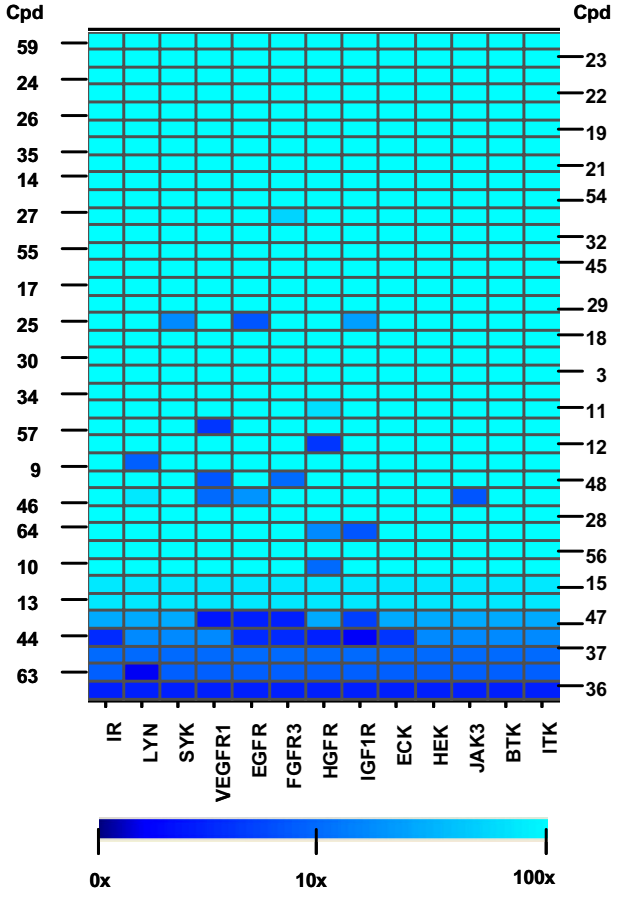


Figure 4. Heat map of selectivity ratio for representative analogs.

Table 5. Cellular and early in vitro ADME profiling

Compound	IL-2 Inhibition[a] IC_{50} (μM)	CYP Inhibition IC_{50} (μM)			HLM, $T_{1/2}$ (min)	Caco-2 AB/BA (10^{-6} cm/s)
		2C9	2D6	3A4 (BFC/BQ)		
3	0.084	1.0	4.3	0.7/1.4	48	4.0/6.9
23	0.14	1.4	4.7	6.9/>30	65	1.6/1.9
57	0.19	8.4	1.7	1.7/13	40	10.6/10.2
61	0.72	17.6	>30	28/>30	66	8.1/10.6

[a] Inhibition of IL-2 production in human $CD4^+$ T cells stimulated with anti-CD3anti-/CD28 mAb.

To address the issue of selectivity we tested analogs in a panel of kinases and found them to be highly selective. Figure 4 shows selectivity data for the 38 representative analogs, with IC_{50} values for PKC-θ <0.5 μM, against a panel of 13 kinases representing data from 494 individual dose responses. Potencies increase upward from the bottom of the figure. Some trends could be visualized even amongst this highly selective set. The analogs where the linker was extended tended to show reduced selectivity (**46**–**48**) as did the *o*-Ph analog **25**. The observed high selectivity of these analogs can be rationalized by the tight requirement for a properly positioned amino group to interact with Asp508 coupled with the 2-benzylamino substituent which controls specificity through its interactions at the glycine-rich loop, the specificity surface, and the hinge regions.

In addition to selectivity, other hit to lead criteria included activity in cells as well as acceptable drug-like properties. The compounds were also shown to be ATP competitive.[11] Cellular activity was assessed by measuring inhibition of IL-2 production in human $CD4^+$ T cells activated by costimulation with anti-CD3 and anti-CD28 mAbs. We also assessed representative compounds for inhibition of CYP's, stability to human liver microsomes (HLM), and permeability in Caco-2 cells (Table 5).[12,13] Although these compounds contain a nitro group which is potential structural alert, the overall profile was acceptable for further advancement in the hit to lead process.

It has been shown that suitably substituted 5-nitro-2,4-diaminopyrimidines are highly potent and selective inhibitors of PKC-θ. The potencies ranged from micromolar to low nanomolar. An arylalkyl moiety is preferred for selectivity at the 2-position but there is a high degree of flexibility in the substitution on the aryl ring. Sterics at the 2-position are also limiting for retaining potency and selectivity. There is a requirement for a suitably spaced basic diamine at the 4-position. Substitution of the terminal amine is permitted provided basicity remains intact. The SAR presented shows that selectivity can be achieved in this known class of kinase inhibitors. We demonstrated that these compounds possess acceptable in vitro metabolic profiles. These compounds were of further interest and additional studies to gain better understanding of the binding mode and the observed selectivity, to expand SAR including 5-nitro replacements, as well as advancing the overall profile of these compounds will be the subject of future reports.

Acknowledgments

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8. Preparation of **24** is representative: compound **I** (12 g 62 mmol) was dissolved in AcOH and cooled to 0 °C. KSCN (6.3 g, 65 mmol) was added portionwise and the mixture was stirred for 2 h. The mixture was diluted with H_2O and filtered. The solid was washed with Et_2O to yield **II** (9.5 g 71%). Compound **II** (100 mg, 0.46 mmol) was suspended in 1 mL abs EtOH. *o*-Bromobenzylamine·HCl (103 mg, 0.46 mmol) was dissolved in 1 mL abs EtOH and added to the reaction mixture quickly at rt. Then Et_3N (129 μL, 0.92 mmol) was added dropwise. The reaction mixture was stirred 16 h. The resulting precipitate was filtered providing 136 mg of the desired (2-bromo-benzyl)-(5-nitro-4-thiocyanato-pyrimidin-2-yl)-amine as a white solid. 1,4-Cyclohexanebis(methylamine) (65 mg, 0.46 mmol) was dissolved in 1 mL CH_2Cl_2 and added to a suspension of (2-bromo-benzyl)-(5-nitro-4-thiocyanato-pyrimidin-2-yl)-amine (40 mg, 0.11 mmol) in 1 mL CH_2Cl_2. The reaction was shaken 16 h at rt. The reaction mixture was then loaded directly onto a 4.5 g pre-packed disposable flash cartridge and eluted with a gradient solvent system (CH_2Cl_2/10% NH_4OH in MeOH). Compound **24**,

43 mg, 89% was isolated as a yellow solid; MS(M+H) = 449 mp 96–100.
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10. Inhibition of enzymatic activity of PKC-θ was measured in a two-step competition fluorescence polarization (FP) assay utilizing Panvera's protein kinase assay kit (PanVera P2940). In the kinase reaction step, 50 nM PKC-θ (Panvera P2996) diluted in assay buffer (20 mM Hepes, pH 7.6 , 0.1 mM $CaCl_2$, 10 mM $MgCl_2$, 0.01% Chaps, 200 μM TCEP (Pierce # 77720), 100 μM Na orthovanadate, and Protease Inhibitor Cocktail (Boehringer Mannheim 1836153; 1 tablet to 50 mL buffer)) is pre-incubated with compound dilutions at rt for 10 min. The reaction is started with a mixture of peptide substrate (RFARKGSLRQKNV, Panvera P2760) and ATP (final assay concentrations are 1 μM peptide and 10 μM ATP). The assay plates are incubated for 60 min at rt. In the FP detection step, fluorescein-labeled phosphopeptide tracer and anti-phosphoserine antibody diluted in quench buffer are added to reaction mixture and incubated for 90 min at rt. FP is measured on a LJL Analyst using fluorescein filters (485 nm excitation, 530 nm emission, 505 nm dichroic).
11. Data not shown; Compound **3** IC_{50} = 0.29 μM at 50 μM ATP and IC_{50} = 0.63 μM at 100 μM ATP.
12. Inhibition of IL-2 production was determined by isolating human CD4+ T cells from whole blood by positive selection. Purified T cells are activated through the TCR and CD28 via anti-CD3 and anti-CD28 mAbs. Compounds are diluted in 5% DMSO to a final concentration of 0.125% DMSO at every dose. 50,000 cells are added in 100 μL of media/well followed by 100 μL of compound or DMSO alone. Cells are incubated overnight at 37 °C and then the supernatants are analyzed for IL-2 with the R&D Systems IL-2 ELISA kit (Cat#02050) following a 1:10 dilution.
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